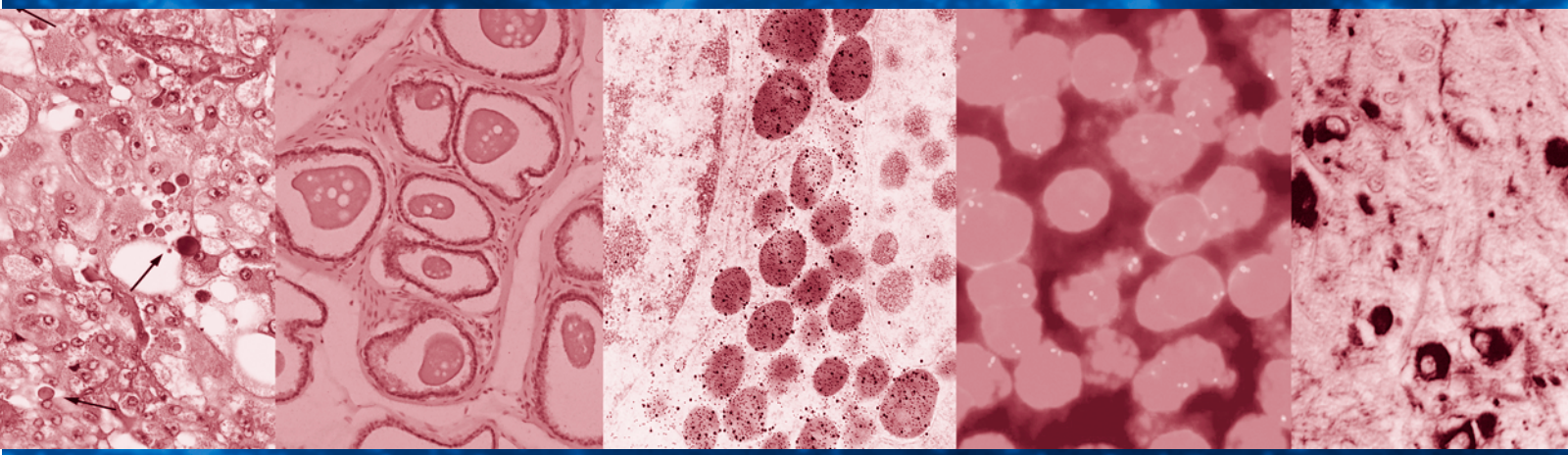
Endocrine Pathology

Differential Diagnosis and Molecular Advances

Edited by

Ricardo V. Lloyd, MD, PhD



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ENDOCRINE PATHOLOGY

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DIFFERENTIAL DIAGNOSIS AND MOLECULAR ADVANCES

Edited by

RICARDO V. LLOYD, MD, PhD
Department of Pathology
Mayo Clinic, Rochester, MN

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Preface

Endocrine Pathology: Differential Diagnosis and Molecular Advances provides a broad overview of the major areas of endocrine pathology using the general approach practiced by diagnostic pathologists. Differential diagnoses based on the gross, and especially the histopathologic, aspects of the lesions are emphasized. Recent developments in the classification of various tumor types are used in the description of endocrine tumors in different organs and tissues.

The field of endocrine pathology is in rapid transition. Most of the chapters attempt to capture these dynamic changes by discussing the most significant developments in cell and molecular biology that help in understanding the pathophysiology of disease processes and have the potential of contributing to the diagnostic aspects of endocrine pathology. In certain of these areas in which there has been a virtual explosion of knowledge, separate chapters have been dedicated to cover these recent developments.

The first chapter of *Endocrine Pathology: Differential Diagnosis and Molecular Advances* lays the groundwork for understanding many of these recent developments with a detailed review of basic cell and molecular biology. Understanding the technical basis of these procedures will allow the reader to be critical in evaluating data generated with these new techniques.

We have included chapters on the treatment of endocrine disorders, especially tumors, from the perspective of the surgeon, medical oncologists, and radiation therapists who have major roles in the treatment of patients with endocrine disorders. These chapters emphasize the importance of the broad perspectives that the endocrine pathologist must have in making specific tissue diagnoses.

The last chapter on future directions provides some clues about future developments in endocrine pathology from the perspective of one author. However, alert readers will quickly decide for themselves the directions that new knowledge will come from to answer difficult diagnostic questions as they read the book.

Today's state-of-the-art concepts will soon become obsolete with new developments in cell and molecular biology. However, the basic time-tested concepts in diagnostic endocrine pathology should remain relevant and continue to serve as the springboard from which the most important new discoveries in this field will be launched over the next few years.

***Ricardo V. Lloyd,** MD, PhD*

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Contributors

KIRSTEN A. ATKINS, MD, *Department of Pathology, Medical College of Virginia, VCU Health System, Richmond, VA*

CESARE BORDI, MD, *Professor of Pathology, Department of Pathology and Laboratory Medicine, Section of Anatomic Pathology, University of Parma, Parma, Italy*

GIANNI BUSSOLATI, MD, FRCPath, *Section of Pathological Anatomy, Department of Biomedical Sciences and Human Oncology, University of Turin Medical School, Turin, Italy*

JOAQUIM CALAF, MD, *Department of Gynecology, Hospital Santa Creu i Sant Pau, Universitat Autònoma de Barcelona, Barcelona, Spain*

CARLO CAPELLA, MD, *Servizio di Anatomia Patologica, Universitá dell'Insubria e Ospedale di Circolo, Varese, Italy*

LIANG CHENG, MD, *Department of Pathology, Indiana University School of Medicine, Indianapolis, IN*

TIZIANA D'ADDA, BScD, *Department of Pathology and Laboratory Medicine, Section of Anatomic Pathology, University of Parma, Parma, Italy*

NOBURU EGASHIRA, MD, *Professor of Pathology, Tokai University School of Medicine, Kanagawa, Japan*

ROBERT E. EMERSON, MD, *Department of Pathology, Indiana University School of Medicine, Indianapolis, IN*

JUAN J. ESPINÓS, MD, *Department of Gynecology, Hospital Santa Creu i Sant Pau, Universitat Autònoma de Barcelona, Barcelona, Spain*

ROBERT L. FOOTE, MD, *Division of Radiation Oncology, Mayo Foundation, Rochester, MN*

DANIELA FURLAN, BScD, *Servizio di Anatomia Patologica, Universitá dell'Insubria e Ospedale di Circolo, Varese, Italy*

H. FUTAMI, MD, PhD, *Growth Factor Division, National Cancer Center Research Institute, Tokyo, Japan*

H. RUBÉN HARACH, MD, FRCPath, *Pathology Service,"Dr. A. Onativá" Endocrinology and Metabolism Hospital, Salta, Argentina*

TIMOTHY J. HOBDAY, MD, *Division of Medical Oncology, Mayo Foundation, Rochester, MN*

EVA HORVATH, PhD, *Department of Pathology, St. Michael's Hospital, Toronto, Ontario, Canada*

TORU KAMEYA, MD, *Pathology Division, Shizouka Cancer Center Hospital and Research Institute, Shizouka, Japan*

ASHRAF KHAN, MD, FRCPath, *Department of Pathology, University of Massachusetts Medical Center, Worcester, MA*

PAUL KOMMINOTH. MD, *Institute of Pathology, Department of Medical Services, Kontonsspital Baden, Baden Switzerland*

KALMAN KOVACS, MD, PhD, *Department of Pathology, St. Michael's Hospital, Toronto, Ontario, Canada*

SUNIL KRISHNAN, MD, *Division of Radiation Oncology, Mayo Foundation, Rochester, MN*

STEFANO LA ROSA, MD, *Servizio di Anatomia Patologica, Ospedale di Circolo, Varese, Italy*

RICARDO V. LLOYD, MD, PhD, *Division of Anatomic Pathology, Mayo Clinic, Rochester, MN*

M. BEATRIZ S. LOPES, MD, *Neuropathology, University of Virginia Health Science Center, Charlottesville, VA*

XAVIER MATIAS-GUIU, MD, PhD, *Department of Pathology, Hospital Universitari Arnau de Vilanova, Universitat de Lleida, Barcelona, Spain*

ANNE MARIE MCNICOL, MD FRCPath, *University Department of Pathology, Royal Infirmary, Glasgow, Scotland, UK*

TAKUYA MORIYA, MD, PhD, *Department of Pathology, Tohoku University School of Medicine, Sendai, Japan*

TORAICHI MOURI, MD, *Mouri Clinic, Natori, Miyagi, Japan*

OSAMU MURAKAMI, MD, *Department of Medicine, Tohoku University School of Medicine, Sendai Miyagi, Japan*

YURI NIKIFOROV, MD, PhD, *Department of Pathology, University of Cincinnati, Cincinnati, OH*

VÂNIA NOSÉ, MD, PhD, *Department of Pathology, Children's Hospital, Boston, MA*

ANNA B. O'GRADY, MD, *Cytopathology Section, National Cancer Institute Center for Cancer Research, Bethesda, MD*

ROBERT Y. OSAMURA, MD, *Professor of Pathology, Tokai University School of Medicine, Kanagawa, Japan*

DONATELLA PACCHIONI, MD, *Section of Pathological Anatomy, Department of Biomedical Sciences and Human Oncology, University of Turin Medical School, Turin, Italy*

MAURO PAPOTTI, MD, *Section of Pathological Anatomy, Department of Biomedical Sciences and Human Oncology, University of Turin Medical School, Turin, Italy*

AUREL A. PERREN, MD, *Department of Pathology, University of Zurich, Zurich, Switzerland*

SILVIA PIZZI, PhD, *Section of Anatomic Pathology, Department of Pathology and Laboratory Medicine, University of Parma, Parma, Italy*

CELESTE N. POWERS, MD, PhD, *Department of Pathology, Medical College of Virginia, VCU Health System, Richmond, VA*

GUIDO RINDI, MD, PhD, *Professor of Pathology, Section of Anatomic Pathology, Department of Pathology and Laboratory Medicine, University of Parma, Parma, Italy*

JOSEPH RUBIN, MD, *Division of Medical Oncology, Mayo Foundation, Rochester, MN*

ANNA SAPINO, MD, *Section of Pathological Anatomy, Department of Biomedical Sciences and Human Oncology, University of Turin Medical School, Turin, Italy*

HIRONOBU SASANO, MD, *Department of Pathology, Tohoku University School of Medicine, Sendai, Japan*

BERND W. SCHEITHAUER, MD, *Department of Pathology, Mayo Clinic, Rochester, MN*

FAUSTO SESSA, MD, *Servizio di Anatomia Patologica, Universitá dell'Insubria e Ospedale di Circolo, Varese, Italy*

TAKASHI SUZUKI, MD, PhD, *Department of Pathology, Tohoku University School of Medicine, Sendai, Japan*

KAZUHIRO TAKAHASHI, MD, *Associate Professor of Molecular Biology and Applied Physiology, Tohoku University School of Medicine, Sendai Miyagi, Japan*

GEOFFREY B. THOMPSON, MD, *Department of Surgery, Mayo Foundation, Rochester, MN*

T. TSUKADA, MD, *Growth Factor Division, National Cancer Center Research Institute, Tokyo, Japan*

THOMAS M. ULBRIGHT, MD, *Department of Pathology, Indiana University School of Medicine, Indianapolis, IN*

SERGIO VIDAL, PhD, *Department of Pathology, St. Michael's Hospital, Toronto, Ontario, Canada*

MARCO VOLANTE, MD, *Section of Pathological Anatomy, Department of Biomedical Sciences and Human Oncology, University of Turin Medical School, Turin, Italy*

AXEL WALCH, MD, *Institute of Pathology, University of Freiburg, Ludwig-Aschoff-Haus, Freiburg, Germany*

MARTIN WERNER, MD, *Institute of Pathology, University of Freiburg, Ludwig-Aschoff-Haus, Freiburg, Germany*

K. YAMAGUCHI, MD, *Shizouka Cancer Center Hospital and Research Institute, Shizouka, Japan*

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1 Methods in Cellular and Molecular Pathology

PAUL KOMMINOTH, MD, AUREL A. PERREN, MD, AXEL WALCH, MD, AND MARTIN WERNER, MD

INTRODUCTION

In the last two decades immunohistochemical and molecular techniques have significantly contributed to our understanding of the function, differentiation, and oncogenesis of endocrine cells as well as tumor growth and biologic behavior of endocrine tumors. The purpose of this chapter is to provide an overview and introduction to some cellular and molecular methods that have been applied in diagnostic and investigative endocrine pathology. General principles and possible applications of each method are outlined, but it is beyond the scope of the chapter to provide detailed protocols. For in-depth information about specific applications we refer to publications in the literature that are cited in the text.

In the first part, immunohistochemical techniques that allow for the detection of specific cellular components and products are discussed. The second part of the chapter deals with molecular methods that are applied *in situ* to identify DNA, specific mRNAs, and chromosomal structures at a cellular level, such as *in situ* hybridization (ISH) and fluorescent *in situ* hybridization (FISH). The third part of the chapter focuses on liquid-based molecular methods that are applied in endocrine pathology. These methods include microdissection of cell groups for molecular analyses, polymerase chain reaction (PCR)–based methods for DNA and RNA detection, as well as the analysis of mutations, loss of heterozygosity, and clonality. Comparative genomic hybridization (CGH) and the array technology to identify chromosomal gains and losses as well as gene expression are also discussed.

IMMUNOHISTOCHEMISTRY

Immunohistochemical techniques, introduced by Coons et al. in 1942 [1], have greatly facilitated the phenotyping of cells and tumors in diagnostics and research and have offered new objective criteria for diagnosis and classification of endocrine diseases and tumors. Today it is possible to analyze a large variety of antigens, not only in fresh but also in formalin-fixed and paraffin-embedded tissues or cells.

PRINCIPLES Immunohistochemistry is a method that is based on the specificity and affinity of antibodies for the detection and precise localization of epitopes in a tissue section or cell preparation. An epitope is a specific antibody binding site of about 5–10 amino acids. The technique of immunohistochemistry is in principle composed of two steps. In a first step, a so-called *primary antibody* is applied, which binds specifically to the epitope of a particular antigen in the tissue or cell. In a second step the binding between antigen and antibody is visualized using direct or indirect detection techniques.

ANTIBODIES Antibodies can be raised to a wide range of cellular entities such as specific cellular products, parts of the cytoskeleton, adhesion molecules, receptors, molecules of the extracellular matrix, and even infectious agents.

Two types of antibodies are applied for immunohistochemistry: polyclonal antisera and monoclonal antibodies. *Polyclonal antisera* are produced by immunizing animals such as rabbits, goats, sheep, and donkeys with a purified antigen to stimulate antibody production. After immunization the periodically harvested sera of the animals can be used for the immunohistochemical method. Polyclonal antisera exhibit a high avidity, but may show crossreaction to other epitopes in tissues and cells, leading to unwanted background reactions. *Monoclonal antibodies*, in contrast, are generated by the fusion of myeloma cells with immunoglobulin-producing spleen cells of immunized animals. The fusion leads to an immortalization of cell hybrids, which continuously produce the specific antibody in culture, which is harvested from the culture media [2,3]. Monoclonal antibodies are specific only to one particular epitope, thus leading to a high specificity of the immunohistochemical reaction. In addition to the abovementioned systems, there also exist cell-free methods for the production of monoclonal antibodies.

TISSUE PREPARATION AND EPITOPE RETRIEVAL Immunohistochemistry can be performed on cryostat sections of fresh frozen tissues and cells but is more frequently applied to fixed and paraffin-embedded materials in a setting of diagnostic path-

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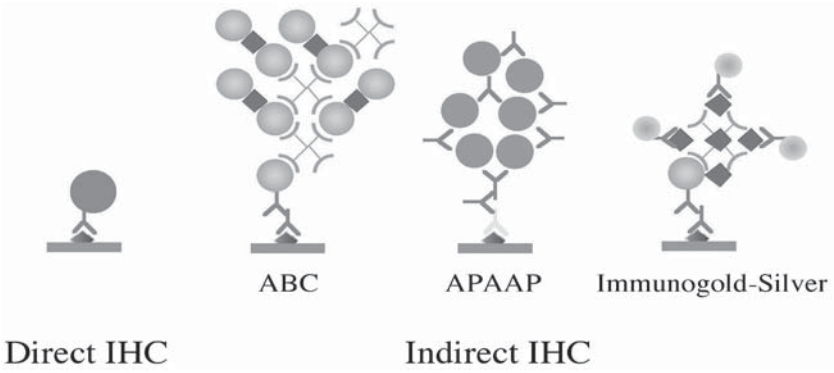


Figure 1 Schematic representation of direct and indirect immunohistochemistry. For abbreviations see text.

ology. Fixation leads to denaturation or precipitation of protein resulting in a masking of antigen structures (epitopes). Thus, it is frequently necessary to pretreat the tissue sections or cells by proteolytic enzymes or to apply heat for antigen and epitope retrieval. *Enzymatic predigestion* of the tissue sections with proteases such as trypsin, pronase, proteinase K, or pepsin has been widely used to retrieve antigens concealed by formaldehyde fixation [4–6]. Epitope retrieval should merely cleave the fixation-caused bonds, resulting in a reconstruction of the original three-dimensional structure of the epitope. Because the cleavage sites of proteolytic enzymes are nonspecific, the epitopes themselves may be affected. Titration of the incubation time and enzyme concentration is needed for different tissue types and fixation times to ensure optimal epitope retrieval and to preserve tissue morphology.

Heat-induced epitope retrieval is able to enhance the reactivity of antibodies that do not benefit from enzymatic pretreatment and can expose epitopes that have been considered to become undetectable by conventional fixation methods. The mechanism of heat-induced antigen retrieval is most probably the loosening of formaldehyde-induced crosslinks, and the heat applied to the sections provides the energy necessary to break crosslinks that have been formed during the formaldehyde fixation between calcium alliance or other bivalent metal cations and proteins. The buffer in which the sections are incubated during the heating process precipitate or chelate the released metal ions. The heating source used to provide the energy to break crosslinks plays only a minor role [7]. Microwave ovens, pressure cookers, wet autoclaves, or electric hotplates can be used. The composition of the buffer in which the sections are submerged during the heating as well as the pH of the buffer solution are of major importance for successful antigen retrieval. Best results are obtained using citrate buffer, Tris-HCl, sodium acetate, and ethylenediaminetetraacetic acid (EDTA) buffers [7]. Most antibodies display a stable behavior without significant variations in the staining intensity over the whole range of pH from 1 to 10. However, some antibodies react best in a particular pH range. For standardized protocols suitable for the majority of antibodies a pH 8–9 is optimal for Tris-HCl, pH 6–7 for citrate buffers [8], and heating for 20–30 min. It is important to note that some antibodies do not profit from this method and display the same, decreased, or even abolished staining reactivity after antigen retrieval (for review *see* [7]). For some rare antibodies a combination of protease pretreatment and heat-induced antigen retrieval has to be combined to reach acceptable results. The best pretreatment for a particular antibody cannot be predicted, and for each new antibody an evaluation of the most effective method has to be evaluated.

IMMUNOHISTOCHEMISTRY METHODS Depending on the detection system, dewaxed, rehydrated, and pretreated slides or cells are preincubated with buffers containing blocking reagents for endogenous peroxidase and nonspecific antibody binding sites. Next the primary, either polyclonal or monoclonal, antibody appropriately diluted in buffer (usually phosphate-buffered saline) is applied and incubated for 30 min up to 24 h in a humidified chamber at room temperature or 4°C to allow antigen–antibody binding. After removal of unbound antibody by a brief wash in buffer, the antigen–antibody binding sites are visualized using direct or indirect methods (Fig. 1). For the *direct detection method*, the primary antibodies have been labeled with a so-called marker molecule (e.g., fluorescent dyes, enzymes, or colloidal gold). Fluorescent dyes can be visualized using ultraviolet or blue light and appropriate filter systems in fluorescence microscopy and binding sites of antibodies, which are coupled to enzymes, by adding colorless colorimetric substrates (chromogens), which are converted into precipitating dyes visible in transmission microscopy. Fluorescent dyes are not widely used in diagnostics because of the need of fluorescent microscopy and problems associated with the correlation of antigen expression and morphology.

For the *indirect detection methods* the antigen–antibody reaction sites are visualized by using additional immunological or chemical reaction steps. Three labeling and detection systems have reached broad acceptance in diagnostic pathology. The enzyme *horseradish peroxidase* (HRP), which can be conjugated to antibodies and other reagents, is the most widely used immunolabeling system. Using H2O2 as substrate, it can convert chromogens such as diaminobenzidine (DAB; brown), aminoethylcarbazole (AEC; red), and others into visible precipitates in tissue sections, which are permanent in synthetic mounting media. A problem with HRP-conjugated antibodies is that endogenous enzyme within tissues (e.g., red blood cells, polymorphic leukocytes, macrophages) will also yield a positive signal. Pretreatment of the tissue sections and slides with hydrogen peroxide can satisfactorily reduce the background

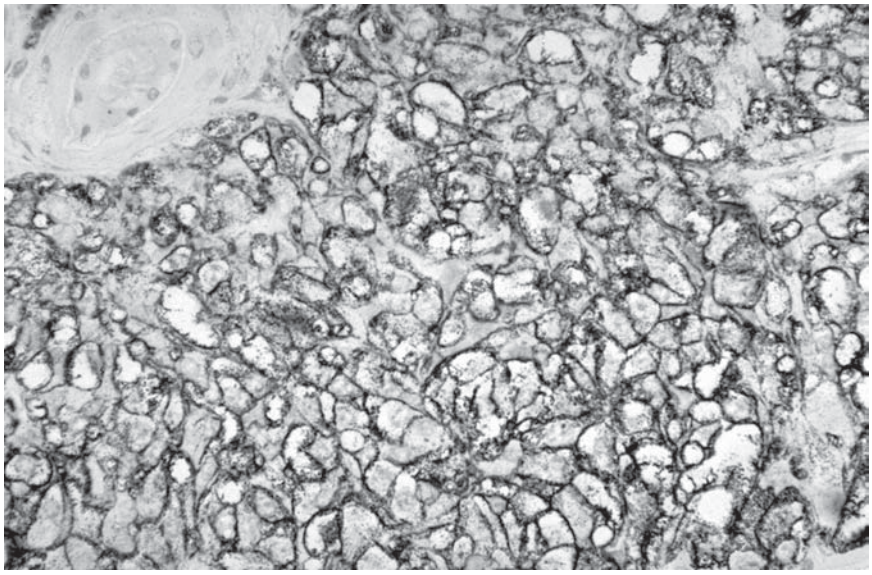


Figure 2 Immunohistochemical detection of polysialic acid in a medullary thyroid carcinoma using immunogold technique with photochemical silver amplification. Note the strong cell membrane staining of tumor cells.

signal. Another important enzyme used in immunohistochemistry is *alkaline phosphatase* (AP). In this system, there is no need to block endogenous enzyme activity prior to incubation, as this may be achieved by the addition of levamisole to the substrate medium. This is necessary especially in gut and placenta, where high levels of endogenous enzyme are present. A problem of the alkaline phosphatase system is that the slides have to be mounted in aqueous media.

The third method is the *immunogold technique*, which can also be applied to electron microscopy [9–11]. Gold-labeled secondary antibodies or complexes exhibit a reddish signal in light microscopy and the reaction can be enhanced using photochemical silver amplification. The silver-intensified gold particle labeling results in a nondiffusible, permanent staining and sections can be counterstained with routine methods and mounted in xylene-based mountains [4,12,13] (Fig. 2). The intense reaction makes the immunogold method valuable for morphometric studies using image-analysis equipment. Because the technique is not prone to false-positive signals by endogenous enzymes or binding molecules, it is the label of choice for immunohistochemistry in "difficult organs" such as kidney, gut, and liver [14].

Several variations of indirect detection methods have been developed in recent years. All are aimed to yield an amplification of the detection signal. The most frequently applied modifications include (1) the peroxidase–antiperoxidase (PAP) or alkaline phosphatase–anti-alkaline phosphatase (APAAP) technique, based on immunological binding affinities, and (2) the avidin–biotin complex (ABC) technique, which is based on chemical affinity. The PAP [15] and APAAP systems [16] make use of a soluble immunocomplex that is formed from the natural affinity between an enzyme label and an antibody against that enzyme. The antibody used in the complex has to be raised in the same species as the primary antibody to enable a secondary antibody (link or bridging antibody) to bind the two together. The link antibody has to be used in excess so that it can both bind the primary antibody and the antibody that forms the immunocomplex. As a three-step procedure, both methods are very sensitive, and by repeating the second and third layers sensitivity can even be improved. ABC methods [17] are based on the natural affinity of avidin and streptavidin for biotin, which is bound in up to four molecules to each avidin molecule (Fig. 3). In addition, in this method a link antibody that is covalently bound to biotin has to be applied. There is a potential problem of avidin binding to endogenous biotin, particularly in liver and kidney as well as in frozen sections. It can be reduced by preabsorption of the tissue sections in avidin followed by biotin.

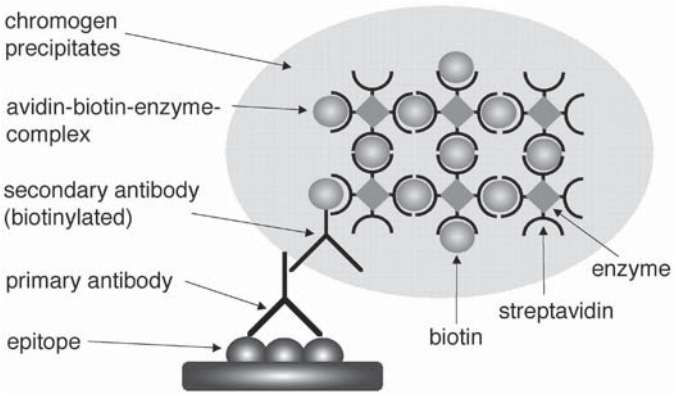


Figure 3 Schematic representation of the ABC method.

For the different enzymatic labels, several chromogens are available. The most widely used chromogens, among others, include DAB (brown) (Fig. 4) and AEC (red) for the detection

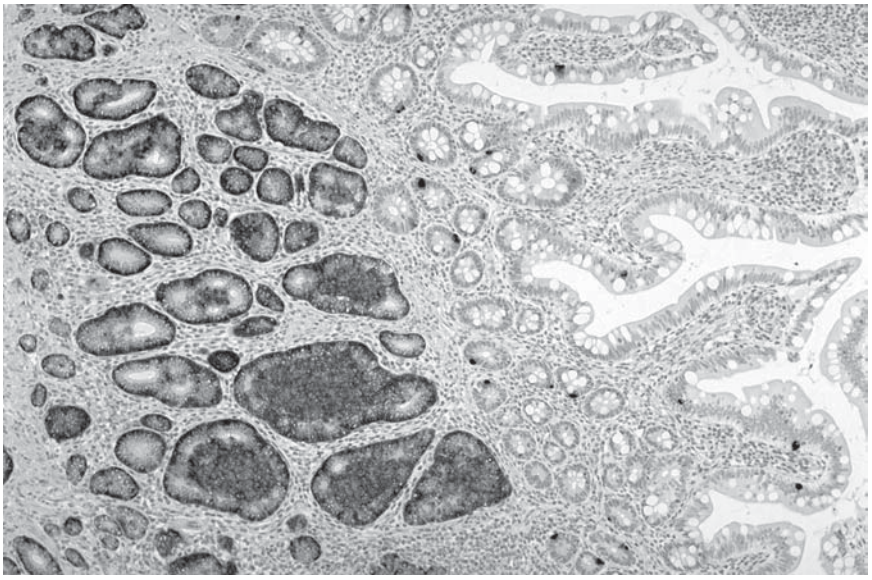


Figure 4 Immunohistochemical detection of synaptophysin in a well-differentiated neuroendocrine tumor (carcinoid) of the terminal ileum using the ABC method. Note the labeling of endocrine cells in the non-neoplastic mucosa.

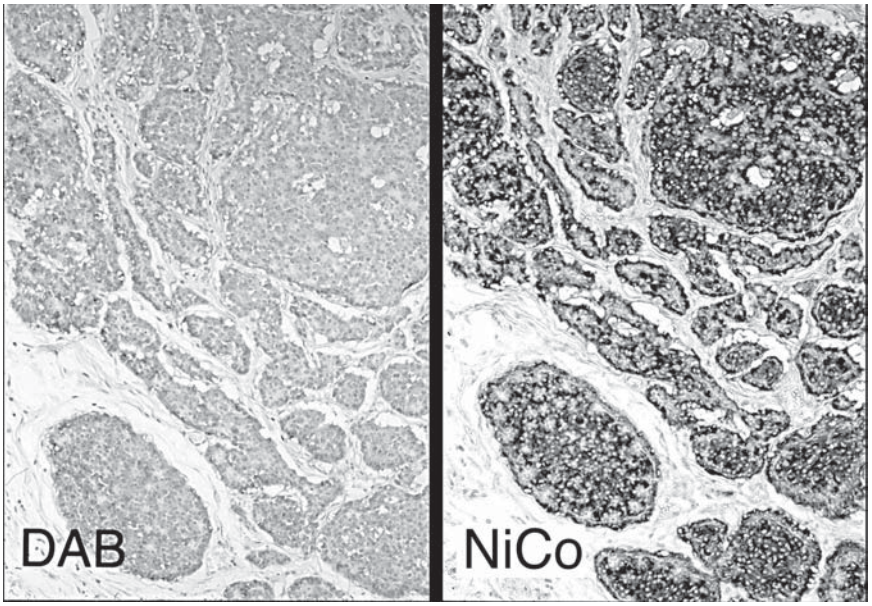


Figure 5 Immunohistochemical detection of synaptophysin in a well-differentiated neuroendocrine tumor (carcinoid) of the terminal ileum using the ABC method with DAB as chromogen (**left**; DAB) and nickel–cobalt intensification of the DAB signal (**right**; NiCo).

of HRP, and fast red or new fuchsin for AP activity. When long incubation times for AP substrates are required (e.g., in nonradioactive, immunobased detection systems of *in situ* hybridization with digoxigenin-labeled probes), the chromogen 5-bromo-4-chloro-indoxylphosphate tetrazolium (NBT-BCIP) is used [18].

SIGNAL INTENSIFICATION AND AMPLIFICATION In recent years, several attempts have been undertaken to increase further the detection sensitivity of immunohistochemistry and to provide detection signals with high contrast. One is a method to intensify DAB reactions by adding an additional step with incubation of the sections in cobalt chloride and nickel salt [19]. The *nickel–cobalt intensification* procedure converts the brown DAB precipitate into a black reaction product that is easily visible with high contrast in tissue sections or cells (Fig. 5). The technique described by Adams [19] is highly sensitive, inexpensive, easy to perform, and produces low background. The magnitude of intensification obtained by this procedure is difficult to assess, but it is estimated to be in the range of 5- to 20-fold. The color modification of DAB by metallic ions can also successfully be applied to double immunostaining procedures. Another recently introduced modification of immunohistochemistry is the biotin amplification technique called the *catalyzed reporter deposition* (*CARD*) method [20,21] (Fig. 6). In our hands the CARD procedure allows a significant increase

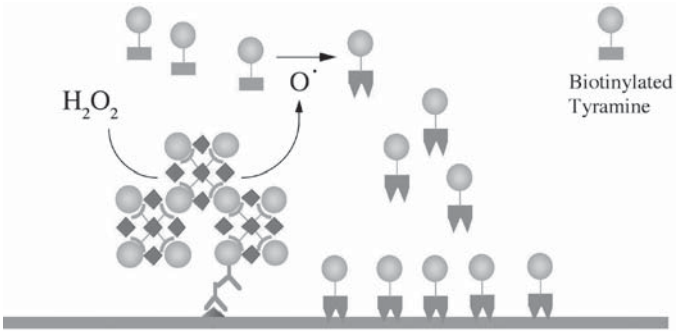


Figure 6 Principle of catalyzed reporter deposition (CARD) using biotinylated tyramine.

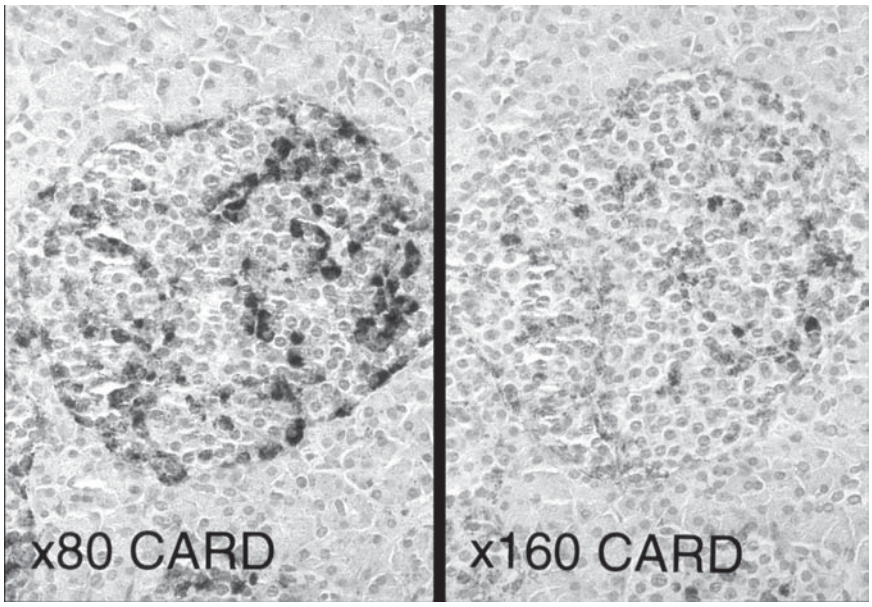


Figure 7 Immunohistochemical detection of glucagon in a pancreatic islet using CARD signal amplification. After 80× dilution of the primary antibody (**left**) a strong signal is still detectable, which is slightly reduced after 160× dilution of the primary antibody (**right**).

in sensitivity of immunohistochemical signals when compared to conventional ABC procedures without production of increased background [7] (Fig. 7). This amplification technique, which has also been applied for ISH [22–24], is based on the deposition of biotinylated tyramide onto proteins attached to the substrate through the catalytic action of HRP. The binding of tyramide to proteins at or near the site of HRP is believed to be due to the production of free oxygen radicals by the enzyme. Activated biotinylated tyramide is attached to covalently bound biotin molecules and other electron-rich moieties, such as tyrosine, phenylalanine, or tryptophan. The biotin sites on the bound tyramide act as further binding sites for, for example, streptavidin–biotin complexes or enzyme/fluorochrome-labeled streptavidin [7].

Another rapid and highly sensitive method to amplify the detection signal, which has been applied to intraoperative frozen sections, is the enhanced polymer one-step staining (EPOS) [25] and two-step system (Envision). In both systems, primary or secondary reporter molecules are directly bound to a large polymer and allow for the rapid one- or two-step detection of epitopes without significant background staining.

DOUBLE AND TRIPLE LABELING The demonstration of two or more antigens in the same tissue section or cell can be achieved by either sequential or simultaneous staining. In the former approach the first antigen–antibody complex is fixed or stabilized prior to the application of the second primary antibody and detection system. In the latter method two or more antigens are visualized at the same time. This can be achieved either by (1) using two or more chromogens or fluorescent labels, (2) using two or more enzyme systems, and (3) a combination of enzyme systems and immunogold labeling (Fig. 8). Ideally, primary antibodies are raised from different species to prevent cross-reactivity. If not possible, two different enzyme labels or combinations of enzyme and immunogold or fluorescent systems have to be used [26]. Triple immunostainings are also available, for example, using an immunogold system for the third immunoreaction.

CONTROLS AND TESTING OF ANTIBODIES To achieve specific immunohistochemical results, a variety of control experiments should be performed in parallel sections, which include (1) absorption of the primary antibody by its purified antigen, (2) incubation of slides with nonimmune serum from the same

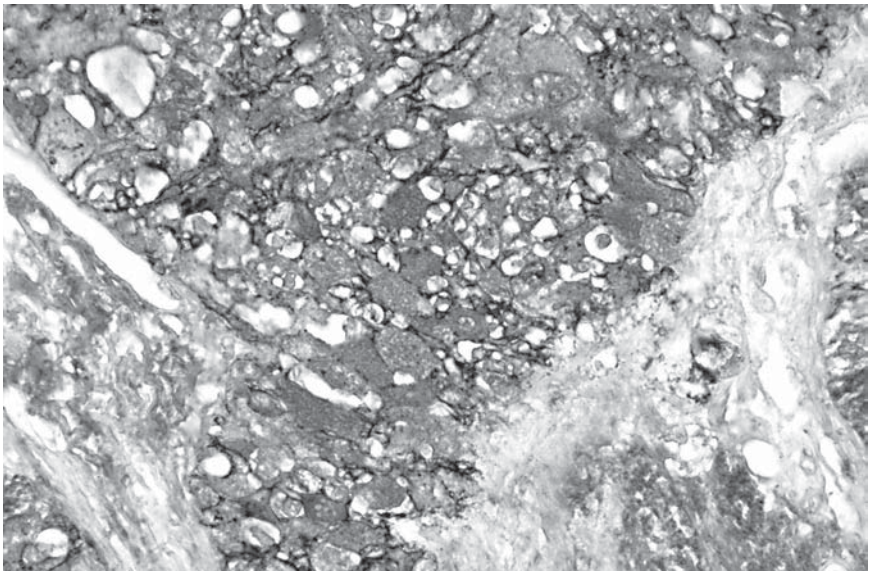


Figure 8 Double immunolabeling of calcitonin and polysialic acid in a medullary thyroid carcinoma. Note the cytoplasmic dark gray staining of calcitonin in the tumor cells and the black cell membrane signal of polysialic acid.

Table 1
Approach for Testing New Antibodies

	Antibody dilution	*Epitope retrieval*	*Blocking steps*	*Immunohistochemical detection*
1	Polyclonal 1:100	None	None	ABC System, DAB
	Monoclonal 1:10	0.01% Trypsin 0.1% Pronase	Defatted 2–4% milk	
2	Increase/decrease dilution	Microwave 20 min 0.01*M* Monohydrate citric buffer, pH 6.0	1% Bovine serum albumin (BSA)	Nickel–cobalt amplification
3			Phosphate-buffered saline (PBS) +++ (1% BSA, 0.05% Triton X-100, 0.05% Tween 20)	Immunogold–silver method
4			Salt concentration in the buffer (0.2 → 0.3 → 0.5 *M*), pH 7.5 → 8.5)	

species in which the primary antibody was raised, (3) omission of the primary antibody, and (4) confirmation of the immunohistochemical results using another independent technique, for example, ISH. Furthermore, positive and negative control samples (tissue with known positivity and negativity for the appropriate antibody used) that have been processed identically to the test tissue should be included in each immunohistochemical procedure. For some antigens and/or samples, however, it may not be necessary to analyze separate control tissues in parallel, as built-in controls (such as normal islets and exocrine tissue adjacent to endocrine pancreatic tumors) may serve as appropriate "internal" positive and negative controls.

The number of available primary antibodies for diagnostic pathology is rapidly increasing. Usually manufacturers provide appropriate protocols and recommendations for antibody dilution as well as the pretreatment of tissue sections and cells. However, in our experience, a standardized, stepwise testing of new protocols should be applied to yield optimal results. Such an approach is proposed in Table 1.

APPLICATIONS OF IMMUNOHISTOCHEMISTRY IN ENDOCRINE PATHOLOGY The introduction of immunohistochemical techniques played an important role in the understanding of the diffuse or dispersed neuroendocrine system. It helped in the identification and classification of their normal distribution and its hyperplasias as well as neoplasias. The new WHO classification also includes immunohistochemical results of hormone expression as well as proliferation activity of tumors [27].

Cells of the diffuse neuroendocrine system share as common features not only the presence of dense-core granules detected by ultrastructural examination, but also the immunohistochemical expression of chromogranin and synaptophysin (Table 2) [5].

The most widely applied *broad-spectrum neuroendocrine markers* are chromogranin and synaptophysin. *Chromogranin* is present in the majority of neuroendocrine cells and neoplasms; however, tumors that are degranulated or contain only a small number of secretory granules (e.g., small cell carcinoma of the lung and Merkel cell carcinomas) usually exhibit only a weak

Table 2
Neuroendocrine Markers for Immunohistochemistry

Markers for neuroendocrine cells (diffuse neuroendocrine system)	
Broad-spectrum markers	Chromogranins (CgA) Synaptophysin (Syn) Neuron-specific enolase (NSE) PGP 9.5 Leu 7 Synaptic proteins (SNAP-25, Rab3a) Neural cell adhesion molecule (NCAM), polysialic acid
Specific markers	
Adrenal medulla	Catecholamines, enkephalin, S100 protein, somatostatin, VIP
Biliary tract	Serotonin, gastrin, somatostatin, PP, substance P
Gastrointestinal tract	Gastrin, somatostatin, serotonin, substance P, PP, VIP
Liver	Serotonin, ACTH, FSH, LH, TSH, α-chain of glycoproteins
Lung	ACTH, calcitonin, gastrin-releasing factor
Skin (Merkel cells)	CK20, calcitonin, VIP
Endocrine pancreas	Insulin, glucagon, pancreatic polypeptide, somatostatin, gastrin, VIP, serotonin, α-chain of glycoproteins
Paraganglia	Catecholamines, enkephalin, S100 protein, somatostatin, VIP, keratin (some)
Parathyroid	Parathyroid hormone (PTH), parathyroid hormone-related protein (PTH-RP)
Pituitary	ACTH, growth-hormone, FSH, LH, prolactin, TSH, α-chain of glycoproteins, calcitonin
Thyroid C cells	Calcitonin, CEA, somatostatin, ACTH
Markers for other endocrine cells (not of the diffuse neuroendocrine system)	
Adrenal cortex	Steroid-metabolizing enzymes, inhibin, D11, keratin, SF1, synaptophysin, vimentin
Gonads (steroid producing cells)	Inhibin, steroid-metabolizing enzymes, vimentin
Thyroid (follicular cells)	Thyroglobulin, keratin, thyroid transcription factor-1 (TTF1)

Partly adapted from [34,35].
VIP, Vasoactive intestinal polypeptide; ACTH, adrenocorticotropic hormone; FSH, follicle stimulating hormone; LH, luteinizing hormone; TSH, thyroid stimulating hormone; PP, pancreatic polypeptide; D11, adrenocortical marker [33]; SF1, steroidogenic factor 1; CEA, carcinoembryonic antigen.

immunoreactivity. The marker *synaptophysin* is a 38-kDa protein that is present in the membrane of presynaptic vesicles. Because its immunoreactivity is independent of the presence of secretory granules, it usually exhibits a higher sensitivity than the marker chromogranin. *Neuron-specific enolase* is another broad-spectrum marker, which it is not very specific because it also reacts with some non-neuroendocrine cells and tumors. But it can be useful in combination with the previously mentioned broad-spectrum markers, owing to its high sensitivity (e.g., for small cell lung cancer). Other less frequently applied broad-spectrum markers are proconvertases, Leu 7, PGP9.5, synaptic proteins (SNAP 25, RAB 3A), and neuroendocrine-specific protein reticulons. A further marker that is expressed in neuroendocrine cells is the neural cell adhesion molecule (NCAM) and its polysialylated form (which is detected by polysialic acid immunoreactivity) [26,28,29].

Neuroendocrine cells and tumors can be characterized further by the expression of *specific markers* listed in Table 2, as well as *proliferation markers* such as MiB-1 and *endothelial markers* such as CD31 (to quantify the proliferation index and visualize angioinvasion, both important criteria in the new WHO classification) [27,30]. However, it is important to note that many neuroendocrine tumors typically exhibit a multihormonal expression pattern, and immunohistochemical profiles are not specific for a particular tumor type [31]. This is especially important when analyzing liver metastases in patients with an unknown neuroendocrine primary tumor. Not infrequently, a peptide hormone responsible for a typical clinical syndrome cannot be identified in the responsible tumor by using immunohistochemical techniques. This may be due to rapid secretion of the product, which is not stored in the cells and thus not detectable by immunohistochemistry. In those circumstances, ISH to detect mRNA can be helpful.

Despite the mentioned limitations, immunohistochemical profiles can be helpful to narrow down a possible primary neuroendocrine tumor [32–35], to distinguish benign from malignant endocrine lesions [30,36], or to provide prognostic and therapeutic information [37]. It is also an important tool in research, for example, to verify overexpression of oncogenes and loss of tumor suppressor genes in endocrine neoplasms [38].

MOLECULAR METHODS

INTRODUCTION TO MOLECULAR BIOLOGY RNA and DNA are composed of nucleotides, which consist of sugar moieties, linked to a phosphate group on carbon 5, and to purine or pyrimidine bases on carbon 1. In DNA there exist four types of bases: adenine, thymine, guanosine, and cytosine (A, T, G, C). In RNA, uracil (U) is substituted for T (Fig. 9). DNA and RNA also differ in the identity of the sugar, with desoxyribose present in DNA and ribose in RNA. DNA is a polymer of nucle-

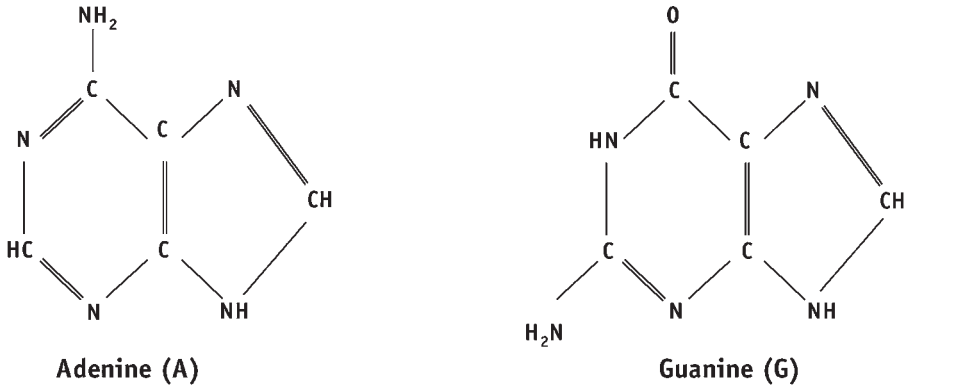


Figure 9 Chemical structure of the common five nucleic acids.

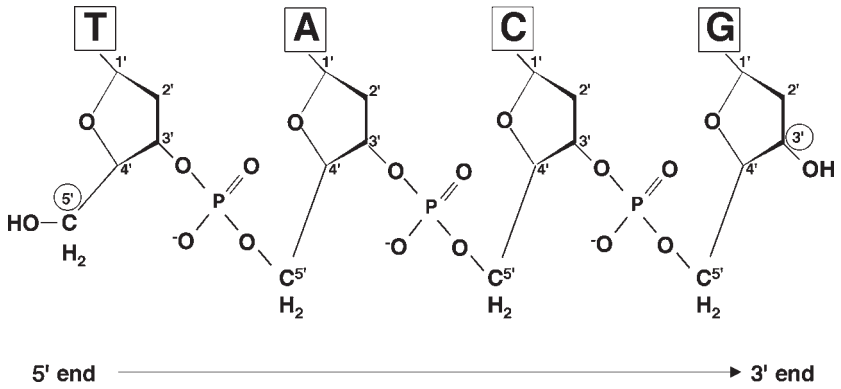


Figure 10 Chain of nucleotides in DNA. Bases (T, A, C, and G) are bound to desoxyribose molecules at carbon atom 1'. Phosphodiester bonds link carbon atom number 3' with number 5' of the successive sugar residues. The so-called 5' end of the DNA strand has a terminal sugar residue in which carbon 5' is not linked to a neighboring sugar and the 3' end one with an unbound carbon 3' (5' → 3' direction of DNA).

otides linked together by phosphodiester bonds between carbon 5 of one sugar and carbon 3 of the following sugar (Fig. 10). Nucleotide sequences of a polynucleotide are always read from the 5' to the 3' direction, as enzymes involved in synthesis of DNA and RNA also work in the 5' to 3' direction. In nature, DNA exists in a double-stranded, double-helix structure. The purine and pyrimidine bases, which stick out from the sugar-phosphate backbone, form hydrogen bonds and stabilize the double-helix conformation. The basepairs formed between A and T are stabilized by two hydrogen bonds, while the pairing between G and C is stabilized by three hydrogen bonds (Fig. 11). The two strands of DNA in a double helix are oriented in opposite directions in a complementary manner.

A *gene* is the functional unit of DNA that is transcribed into RNA, which encodes the amino acid sequence of a protein. During DNA *replication*, the double helix unwinds and serves as two template strands for duplication of the DNA. The human genome is encoded by 3.5 billion nucleotides located on 23 pairs of chromosomes. It is estimated that genes comprise only about 10% of total DNA, encompassing approx 30,000–40,000 functional genes.

RNA is single stranded and identical in sequence (although with U instead of T) to the "sense-strand" of the DNA. The process in which RNA is synthesized from DNA is called *transcription* (Fig. 12). It is synthesized in 5' to 3' direction by RNA polymerases. Within a gene, the information for the amino acid

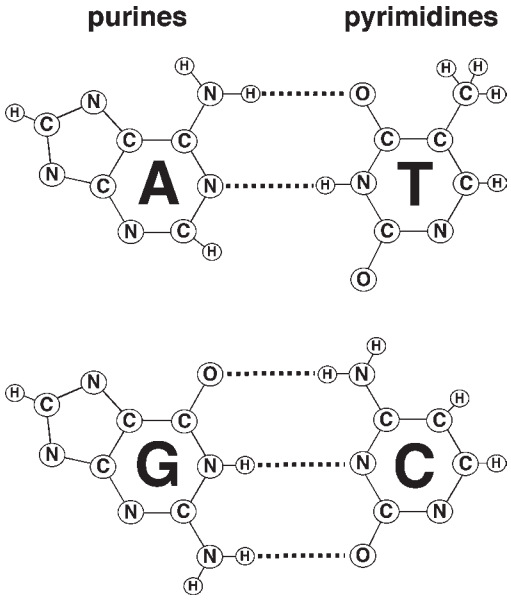


Figure 11 Hydrogen bonding between matching base pairs. There are two bonds between adenine (A) and thymine (T) and three bonds between the guanine (G) and cytosine (C).

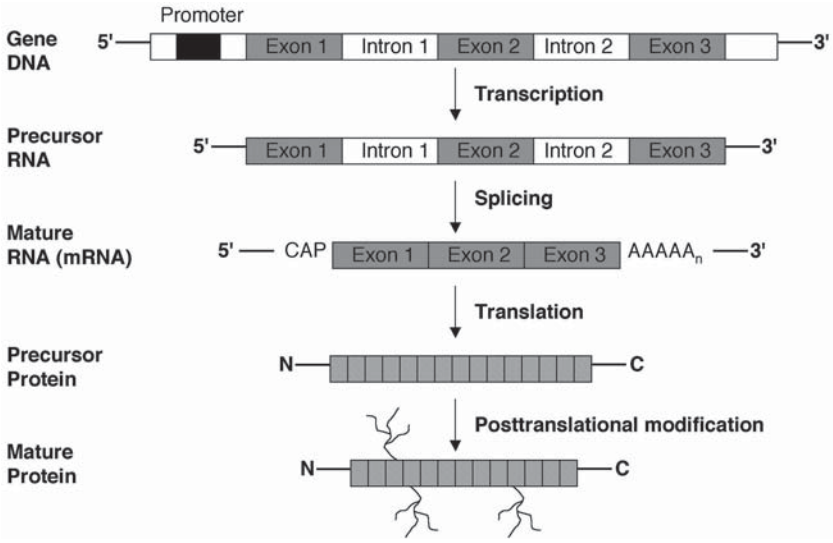
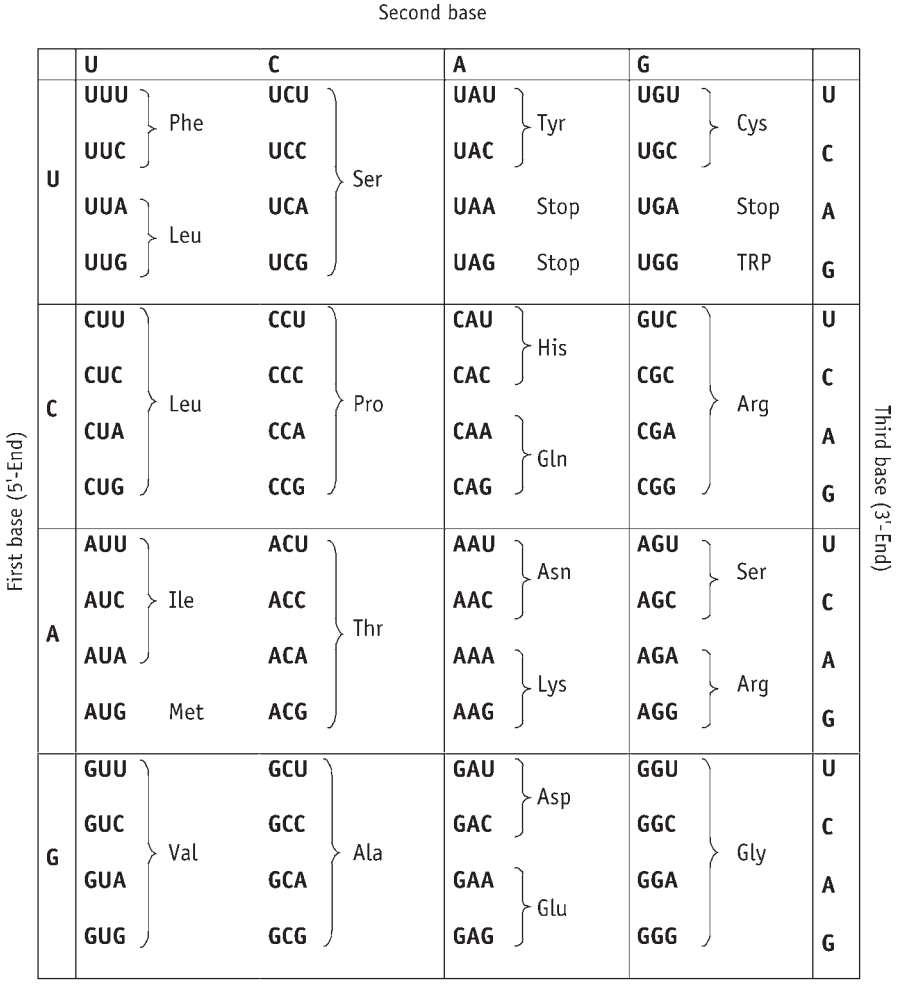


Figure 12 Pathway of gene expression from the gene to protein product in a eukaryotic cell.

sequence (the *coding sequence*) is contained in gene units called *exons*, which are interspersed with noncoding sequence units, termed *introns*. The entire portion of the DNA (including introns and exons) is transcribed into messenger RNA (mRNA). This precursor is then processed into mature mRNA by excision of the intron regions and addition of the 5' cap and a 3' poly(A) tail. The remaining exons are joined together at specific base sequences within the nucleus. The mRNA is then transported to the cytoplasm where its association with the rough endoplasmic reticulum allows its information to be *translated* into a precursor protein product. Proteins are synthesized on ribosomes with the help of transfer RNA (tRNA). Each of the 20 amino acids is linked to a specific tRNA and each tRNA in turn recognizes a complementary three-nucleotide sequence (*codon*) on the mRNA. Thus, mRNAs direct the assembly of amino acid chains in a sequence-specific manner. The 20 naturally occurring *amino acids* can be defined by different codons (Fig. 13), and protein synthesis is initiated by the methoinine codon AUG (start codon) and terminated by the stop codons UAA, UAG, and UGA (Fig. 12). Once translated, precursor protein products are further processed to form the mature gene product, a process that is termed *posttranslational modification* (Fig. 12). A single gene can be responsible for the generation of more than one specific mRNA because of alternate splicing of the precursor RNA so that the mature mRNA represents only a part of the original gene transcript.



Ala	Alanine	Leu	Leucine
Arg	Arginine	Lys	Lysine
Asn	Asparagine	Met	Methionine
Asp	Aspartic acid	Phe	Phenylalanine
Cys	Cysteine	Pro	Proline
Gln	Glutamine	Ser	Serine
Glu	Glutamic acid	Thr	Threonine
Gly	Glycine	Trp	Tryptophan
His	Histidine	Tyr	Tyrosine
Ile	Isoleucine	Val	Valine

Figure 13 The nuclear genetic code.

IN SITU METHODS

***In Situ* Hybridization** ISH is a technique that enables the morphological demonstration of specific DNA or RNA sequences in individual cells in tissue sections, single cells, or chromosome preparations. ISH was introduced in 1969 and has been used primarily for the localization of DNA sequences (39). In more recent years, ISH has also been applied to the localization of viral DNA sequences, mRNA, and chromosomal mapping [18,40,41]. Furthermore, techniques have been adapted for ultrastructural analysis using electron microscopy [42].

Principles ISH is based on the fact that labeled single-stranded fragments of DNA or RNA containing complementary sequences (probes) are hybridized to cellular DNA or RNA under appropriate conditions to form stable hybrids (Fig. 14). The sensitivity of ISH depends on several variables, including the effect of tissue preparation on retention and accessibility of target DNA or RNA, the type of probe construction, efficiency of probe labeling, the sensitivity of the method used for signal detection, and the effect of hybridization conditions on the efficiency of hybridization.

Probes Four main classes of probes are in current use for ISH: (1) double-stranded DNA probes, (2) single-stranded DNA probes, (3) oligonucleotide probes, and (4) single-stranded RNA probes. In principle all types of probes can be employed to localize DNA and mRNA in tissue sections and cells (Fig. 15). The choice of probe depends on several considerations including target nucleic acid, sensitivity and specificity, ease of tissue penetration, stability of probes and hybrids, as well as general

Figure 14 Principles of ISH. A labeled probe binds to the matching complementary sequence of the DNA.

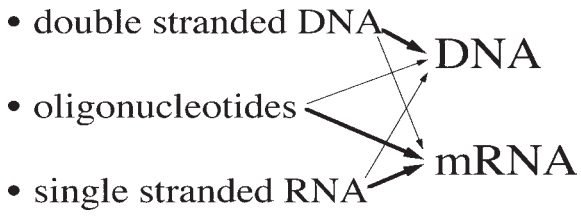


Figure 15 Probe types and possible targets for ISH.

Table 3
Labeling Methods for ISH Probes

• Ds DNA	Random priming
	Nick translation
	PCR
• Oligonucleotides	3' End labeling
	3' End "tailing"
	5' End labeling
• cRNA	In vitro transcription

issues such as laboratory equipment, availability of reagents, and the training of personnel.

Double-stranded DNA probes have found widespread application owing to ease of use, high specific activity, stable hybrids, and relatively high sensitivity due to networking. Disadvantages of those types of probes are reannealing to the probes in solution and the presence of vector sequences (in the case of cloned probes), which can lead to background signals. DNA probes, which have to be denatured before use, can be generated by nick translation, random priming, or PCR in the presence of labeled nucleotides (Table 3). *Nick translation* employs the enzymes DNase I and DNA polymerase I. The 5'-3' exonuclease activity of DNA polymerase I extends the nicks generated by DNase I to gaps and then the polymerase replaces the excised nucleotides with labeled ones. *Random priming* is based on the random annealing of oligonucleotide primers to a linearized and denatured probe followed by synthesis of new DNA along the single-stranded template. The probes are directly labeled during synthesis by the incorporation of nucleotides conjugated to a reporter molecule.

Single-stranded DNA probes can be generated by primer extension on single-stranded templates, by PCR, or by chemical synthesis of oligonucleotides (*see* below). Again, the probes can be directly labeled during synthesis. However, the single-stranded probes have the advantage that reannealing of the probe to the second strand cannot occur. However, this approach has not found broader acceptance in diagnostics and research.

Oligonucleotide probes, typically 20–50 bases in length, can conveniently be tailor-made by automated DNA synthesizers for any nucleic acid sequence published in the literature or available from gene banks. They are relatively inexpensive, exhibit good tissue penetration, allow the generation of discriminating sequences for similar genes and the synthesis of probes from amino acid sequences when the total sequences are not known, and do not require specialized laboratory facilities and personnel familiar with molecular biology methods for cloning, plasmid preparation, and so forth. A disadvantage of oligonucleotide probes is the limited labeling efficiency resulting in a lesser sensitivity when compared to longer nucleic acid probes. Thus, they are not considered suitable for the detection of low-level expressed genes. The limited sensitivity of oligonucleotide probes can be overcome by using a mixture or cocktails of oligonucleotides that are complementary to different regions of the target molecule. Labeling of oligonucleotides is usually performed by either 5'-end labeling with T4 polynucleotide kinase or 3'-end labeling using terminal deoxynucleotidyl transferase [18] (Table 3).

Single-stranded antisense RNA probes are generated using specially constructed and linearized RNA expression vectors or PCR products to transcribe sense or antisense sequences downstream of the appropriate polymerase initiation site (SP6, T7, or T3), which must be present on the vector DNA containing the template (Fig. 16). RNA probes using PCR-generated templates are generated by adding RNA polymerase promoter sequences to the 5'-end of the primers, separated by a spacer. During PCR amplification of the template sequence the appropriate RNA polymerase recognition sites are added to the PCR products, which can be used further for in vitro transcription without knowledge of cloning techniques [43]. The synthesized RNA can be labeled during synthesis by incorporation of ribonucleotides with coupled reporter molecules (in vitro "run off" transcription) (Table 3). The advantages of antisense RNA probes include a constant defined probe size, a high specific activity, a high thermal stability of RNA hybrids, and the possibility to generate sense strand (control) probes. Furthermore, competitive hybridization to the complementary strand, as occurs with double-stranded DNA probes, is excluded and nonhybridized (single-stranded) probes can be removed using RNase digestion after hybridization [18,41].

Labeling Two main types of labeling strategy can be applied: (1) isotopic labeling using ^{3}H, ^{35}S, ^{125}I, ^{32}P, or ^{33}P; and (2) nonisotopic labeling using biotin, digoxigenin, fluorescein, alkaline phosphatase, 5-bromodeoxyuridine, and others (Table 4).

Radioactively labeled probes, as originally described by Gall and Pardue [44], are still widely applied for ISH for several reasons: (1) efficiency of probe synthesis can be monitored by scintillation counters, (2) radioisotopes are readily incorporated into synthesized DNA and RNA using all known enzymes, and (3) autoradiography represents the most sensitive detection system. Signal detection can be achieved with autoradiography employing liquid emulsions. ^{3}H or ^{35}S labeling is most commonly used because of the high resolution of the autoradiographs [18,45]. However, sections hybridized with ^{3}H-labeled probes usually require a rather long exposure (weeks) for signal

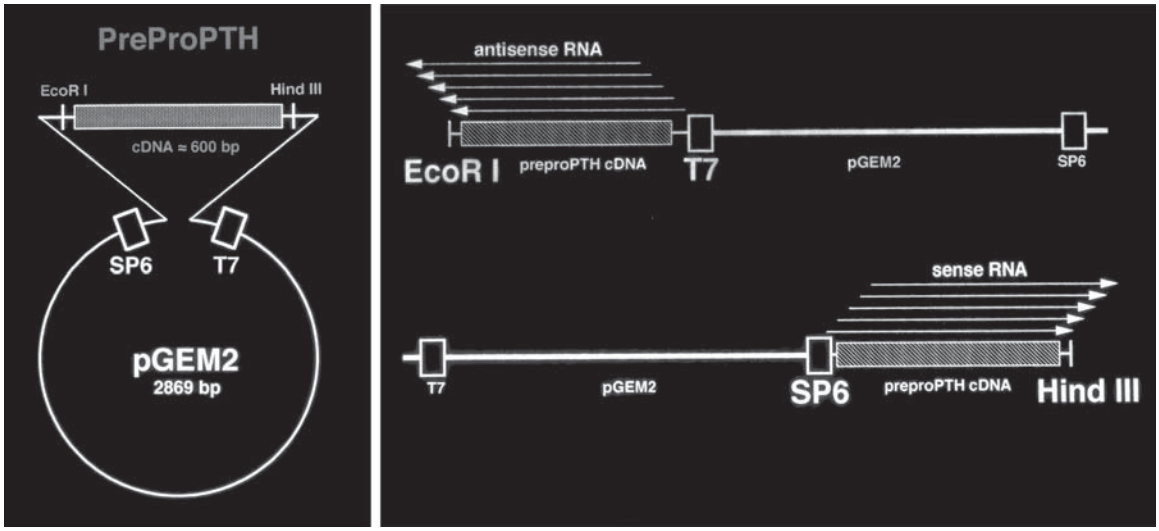


Figure 16 (**Left**) pGEM2 expression vector with preproPTH cDNA cloned into the polylinker site (*EcoR*I and *Hind*III restriction sites). (**Right**) Schematic representation of antisense and sense RNA probe synthesis using in vitro runoff transcription with the linearized plasmid and the appropriate RNA polymerase (T7 for antisense and SP6 for sense RNA).

Table 4
Probe Labels

Isotopic labels
^{3}H, ^{35}S, ^{125}I, ^{32}P, ^{33}P
Nonisotopic labels
Biotin, digoxigenin, fluorescein, alkaline phosphatase, 5-bromodeoxyuridine

detection. If a more rapid detection is desired, labeling with high-energy-emitting radioisotopes such as ^{32}P, ^{33}P, or ^{35}S can yield autoradiographs within days. The use of radioisotopes for ISH is associated with several disadvantages including significant biohazard (requiring special facilities and monitoring for contamination), long exposure time of autoradiographs, limited resolution of signal detection, and limited probe stability, even when stored at –80°C [46]. Furthermore, ISH with isotopic probes is difficult to perform on a routine basis in a nonspecialized clinical laboratory, particularly when analysis must be performed frequently and results obtained quickly.

Nonradioactive probes are much more stable, safer in use, and provide a superior resolution of signal detection together with shorter turnaround times of procedures. Furthermore, the variety of available nonradioactive probe labeling systems provides the opportunity to detect different nucleic acid sequences simultaneously in the same tissue. Visualization of nonisotopic labeled probes can be achieved by histochemistry or immunohistochemistry detection systems, which are well established in most laboratories. The most frequently applied labeling systems in routine laboratories are *biotin* and *digoxigenin* [46]. The latter labeling exhibits a higher sensitivity and less background staining compared to biotinylated probes. *Fluorescent labeling*, either of the probe or of the antibody, has been successfully used for chromosomal ISH or is especially useful for simultaneous detection of different sequences.

Fixation and Pretreatment of Tissue The optimal procedure of *fixation* and tissue preparation should retain the maximal level of cellular target DNA or RNA while maintaining optimal morphological details and allowing a sufficient accessibility of the probe. In contrast to the rather stable DNA, mRNA is steadily synthesized and degraded enzymatically. Consequently, tissue prepared for RNA localization should be fixed or frozen as soon as possible after surgical excision, and the time between excision and adequate fixation has to be taken into account in each case when the results of ISH are interpreted.

Tissues or cells can either be fixed in *protein denaturing fixatives* such as ethanol and acetone or in *crosslinking fixatives* such as buffered formalin and paraformaldehyde. Other fixatives containing picric acid or heavy metals are not suitable for ISH mainly because of nucleic acid destruction. *Pretreatment* of sections with a detergent and/or proteinase digestion is a standard procedure in almost all published protocols to increase probe penetration, particularly in paraffin sections. Commonly, proteinase K or self-digested pronase is used [18]. Alternative methods are microwaving sections in citrate buffer [7,47]. The duration of the proteinase digestion depends on the length of probes (and is not strictly required when oligonucleotide probes are used) and the fixation time of tissues. If possible, standard fixation times should be applied to use standardized protocols; if not possible, a titration of the permeabilization step has to be performed to obtain optimal results. Some protocols use an additional acethylation step with acetic anhydride to reduce nonspecific binding of probes to positively charged amino groups. When working with single-stranded mRNA probes, it is important to avoid degradation, especially of the probe (but also of target nucleic acids) by ribonucleases, which should be inactivated by diethyl pyrocarbonate (DEPC)-treatment of solutions. In many protocols slides and cells are incubated in a cocktail of reagents subsequently used in the hybridization reaction but in the absence of the probe. This *prehybridization* step is intended to saturate sites in the tissue section that might otherwise bind nucleic acids nonspecifically.

Hybridization One of the important advantages of ISH over immunohistochemistry is the fact that the degree of specificity of hybridization reactions can be controlled accurately by varying the reaction conditions. The degree of specificity depends on the construction of the probe used, temperature, pH, concentration of formamide and of salt in the hybridization buffer, the length and GC content of the probe, the extent of sequence identity between the probe and target, and the composition of the washing solution. The "melting" temperature (T_m) of hybrids is the point at which 50% of the double-stranded nucleic acid chains are separated. Depending on the desired stringency and melting temperature of the hybrids, hybridization and washings are undertaken at 15–25°C below the calculated melting temperature. Using very high stringency (e.g., 5°C below melting temperature), it is possible to discriminate between gene sequences of >90% homology [48]. There exist several formulas for calculating T_m and for, for example, oligonucleotide probes, the following formula can be used [48]:

$$T_m = 81.5 + 16.6 \log (\text{molarity of monovalent cations} = \text{sodium concentration}) + 0.41(\%\ \text{GC}) - 675/\text{L}$$
$$(\text{length of probe in bases}) - 0.62\ (\%\ \text{formamide}) - \%\ \text{mismatch}$$

RNA–RNA hybrids usually are approx 10–15°C more stable than DNA–DNA or DNA–RNA hybrids and require more stringent conditions for hybridization and posthybridization washings. For ISH with radioactively labeled dsDNA or cRNA probes usually 2–10 ng of probe and for nonradioactively labeled probes 10–50 ng of probe per section are required. The probe is diluted in the hybridization buffer, 20–30 μL of which is added per section and covered with a coverslip. Slides are placed in a humidified chamber in an oven at 40°C and usually incubated overnight. Most hybridization buffers contain a mixture of 50% formamide, 2× standard saline citrate (1×SSC = 0.15 *M* sodium chloride, 0.015 *M* sodium citrate), dextrane sulfate (usually 10%, to increase the activity of the probe by excluded volume effect), and nonspecific (e.g., salmon sperm) DNA and other bioactive polymers (to enhance binding and to reduce nonspecific background) [18]. In contrast to the localization of mRNA, hybridization to cellular DNA requires the heating of tissue sections (with the hybridization buffer containing the probe) for 5–10 min at 90°C to denature the target (and in case of double-stranded DNA probes also the probe) DNA.

After incubation and removal of the coverslips by incubation into 2× SSC, the nonspecifically bound probe is removed by several *posthybridization washings* using increasing stringency conditions (e.g., 2×, 1×, and 0.5× SSC). When using cRNA riboprobes, high-stringency washings can also be performed in 50% formamide/0.1× SSC at 40°C and nonspecific background signals can further be reduced by treatment of the slides with ribonuclease A (RNase), as the enzyme digests single-stranded but not double-stranded RNA hybrids [45].

Visualization of Signal For *radioactive probes* signal detection is performed by *autoradiography*. Slides are dipped in liquid nuclear track emulsion (in the dark), which are then dried, exposed, and developed. Exposure is carried out at 4°C, as the efficiency of autoradiography is greater than at room temperature. After developing, the tissues are stained with a nuclear stain, mounted, and coversliped (Fig. 17). A high density of silver grains can be observed under bright field illumination; however, a more sensitive means is the use of dark field illumination on a light microscope (Fig. 18). The most common method for visualizing *nonisotopic probes* are histochemical methods using antibodies or chemical compounds directed against the reporter molecule combined with direct or indirect detection systems using fluorescent tags, enzymes (Fig. 19), or immunogold [18].

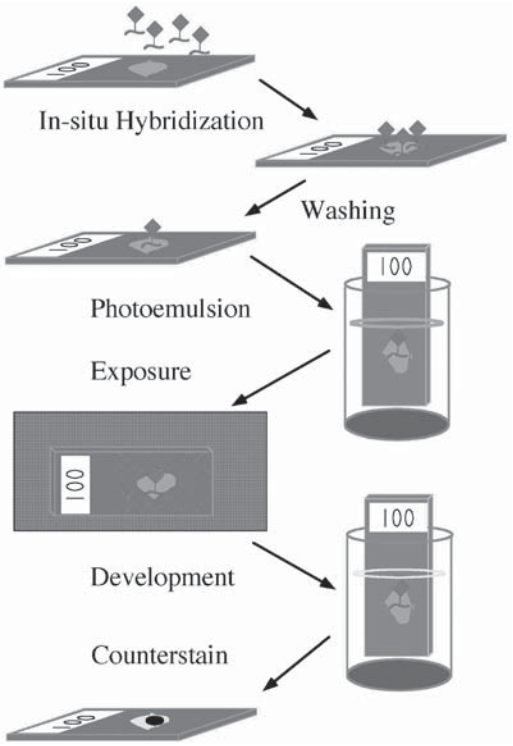


Figure 17 Schematic representation of radioactive ISH.

Controls In order to ensure specific hybridization, a variety of controls have to be performed to detect false-positive and -negative results. In general, results should be confirmed by other molecular or immunohistochemical methods, positive controls (such as tissues or cells known to highly express the gene of interest) and negative controls (such as normal tissues or cell lines that do not express the specific gene), ISH without probe, competition studies with excess of unlabeled specific probe, hybridization with a nonspecific (e.g., viral or vector) sequence, or a sense probe; in addition, sections should be pretreated with RNase or DNase.

Target and Signal Amplification To detect low copies of nucleic acids in tissue sections and cells, several attempts have been made to increase the sensitivity of nonradioactive ISH procedures, such as mixtures of labeled, nonoverlapping oligonucleotides or the application of up to five cytochemical probe detection layers. Recently, several signal and target amplification approaches have been developed, including primed *in situ* (PRINS) DNA synthesis [49], *in situ* PCR, and the CARD system.

Protocols for successful *in situ* PCR have been independently developed by several groups in the late 1980s [50–54]. The principal steps of *in situ* PCR are the following [55]: After

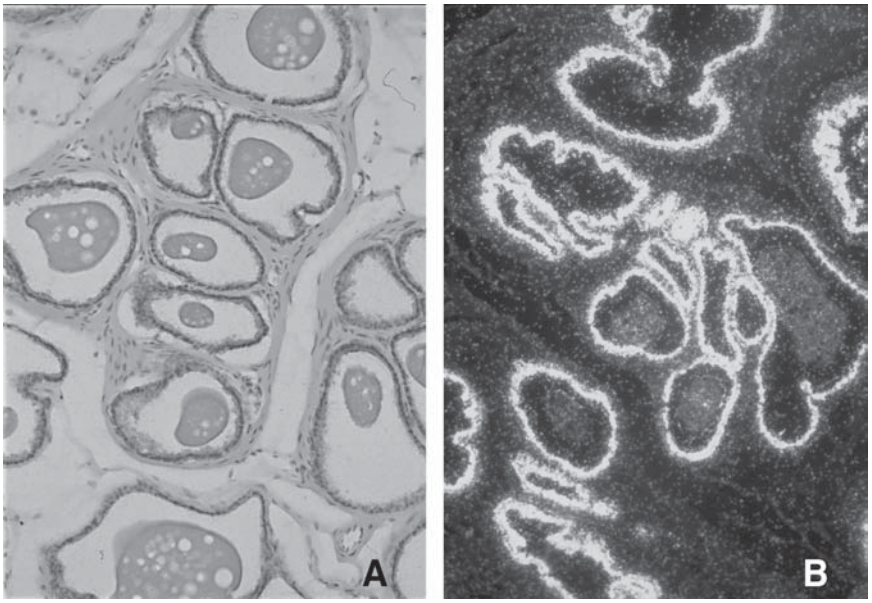


Figure 18 Radioactive ISH using S^{35}-labeled antisense RNA probes for the detection of SVS II in the rat prostate. (**A**) Silver grains over the epithelium of the prostate epithelium are barely visible using bright field microscopy. (**B**) Strong signals are detectable using dark field illumination.

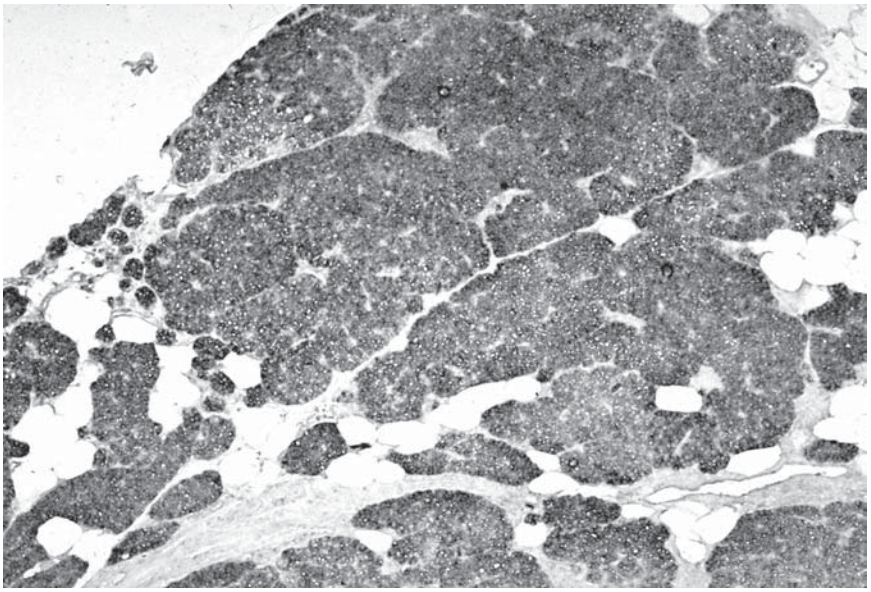


Figure 19 Nonradioactive detection of PTH mRNA in a parathyroid gland using a digoxigenin-labeled preproPTH antisense RNA probe.

fixation and permeabilization of cells and tissues, PCR amplification of target sequences is performed either in intact cells held in suspension in micro-Eppendorf tubes or directly in cytocentrifuge preparations or tissue sections on glass slides. In the former approach the cells are then cytocentrifuged onto glass slides followed by visualization of intracellular PCR products by ISH or immunohistochemistry. *In situ* PCR of cells or tissue sections on glass slides is performed by overlaying the samples with the PCR mixture under a coverslip, which is then sealed with nail polish, rubber cement, or mineral oil to prevent evaporation of the reaction mixture or by using specially designed reaction chambers that are clipped onto the glass slide. The detection of intracellular PCR products is achieved either (1) indirectly by ISH with PCR-product-specific probes (indirect *in situ* PCR), or (2) without ISH through direct detection of labeled nucleotides that have been incorporated into PCR products during thermal cycling (direct *in situ* PCR) [56].

A majority of publications to date have dealt with the detection of viral or foreign DNA within cells. But *in situ* PCR has also been applied to the study of endogenous DNA sequences including human single copy genes, rearranged cellular genes, and chromosomal translocations, to map low-copy-number genomic sequences in metaphase chromosomes and to detect low-copy mRNA and viral RNA [57,58].

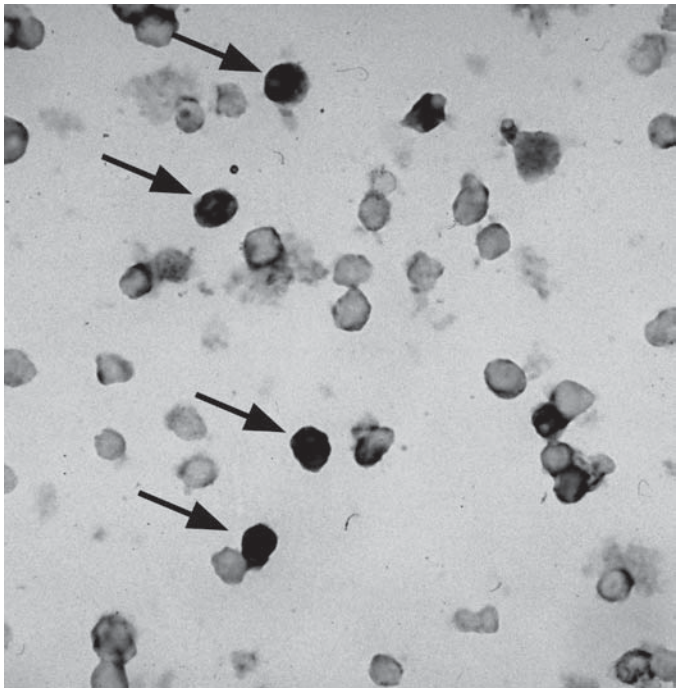


Figure 20 Nonradioactive indirect *in situ* PCR for the detection of a rearranged VH3 DNA sequence in a mixture of rearrangement positive and negative clonal B cells. Note the strong nuclear signal (*arrows*) in rearrangement positive cells and the weak cytoplasmic signal in rearrangement negative cells (possibly caused by so-called diffusion artifacts).

The general principle of *in situ* PCR is simple, and in experimental systems at least the successful *in situ* detection of one copy of cellular (proviral) DNA has been achieved [59]. However, when working with tissue sections prepared from routinely fixed and processed pathology specimens, the success of *in situ* PCR is more limited and prone to frequent false-positive and -negative results. They are mainly linked to poor amplification efficiency and a variety of *in situ* PCR–specific artifacts, which require a multitude of different controls to allow adequate interpretation of results [58,60]. False-positive signals in direct *in situ* PCR mainly result from incorporation of labeled nucleotides into nonspecific PCR products, which not only result from mispriming but also from fragmented endogenous DNA undergoing "repair" by the DNA polymerase ("DNA repair artifacts") or by priming of nonspecific PCR products by cDNA or DNA fragments ("endogenous priming") [56]. Diffusion of PCR products from the site of synthesis to neighboring template negative cells can also lead to false-positive results, a phenomenon that has been termed "diffusion artifact" [61,62] (Fig. 20).

Low amplification efficiency, poor reproducibility, and difficulties in quantitation of results [56,61] have led to a more realistic attitude about the practical potential of *in situ* PCR in recent years [63] and other approaches to target amplification such as *in situ self-sustained sequence replication* (*3SR*) [64, 65] and *in situ transcription* [66] have not found a broader acceptance in the field of *in situ* visualization of mRNA.

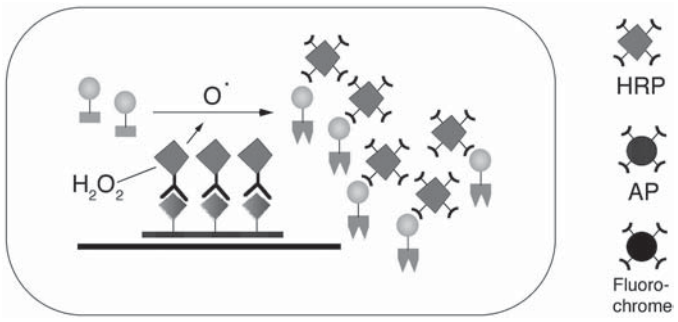


Figure 21 Principles of *in situ* hybridization with CARD signal amplification using digoxigenin-labeled probes and biotinylated tyramides. Horseradish peroxidase (HRP), alkaline phosphatase (AP), or fluorochrome labeled streptavidin can be used for visualization.

Recently, the CARD technique using biotinylated tyramine (tyramide) [20] has successfully been adapted to ISH in cytospins and tissue sections [22–24] as well as to FISH in metaphase and interphase preparations [67]. This signal-amplification technique, which has already been discussed for immunohistochemistry, can significantly increase the sensitivity of DNA and mRNA ISH and appears to be more reliable than the abovementioned target-amplification methods [68] (Fig. 21). It can also be applied on routinely fixed, paraffin-embedded sections and the entire ISH procedure can be shortened to one working day (Fig. 22). We could demonstrate that tyramide conjugates such as digoxigenin-, biotin-, DNP-, TNP-, or fluorescein-tyramides [69,70] provide approximately the same sensitivity, indicating that signal amplification is independent of the tyramide conjugate used [23,24]. Thus, in case of the presence of endogenous (strept) avidin binding sites in the investigated tissue, a tyramide conjugate other than biotin-tyramide can be used for CARD amplification to prevent high background staining.

Applications of ISH in Endocrine Pathology ISH has significantly advanced the study of gene structure and expression at the level of individual cells in complex structural tissues [71–73]. It has contributed substantially to the diagnosis and understanding of neoplastic endocrine diseases [74] and has provided invaluable insights into hormone regulation, storage, and secretion [75,76]. For diagnostic purposes, ISH is most valuable in situations where (1) endocrine tumors show little or no hormone immunoreactivity due to ineffective translation, rapid secretion, or posttranslational modifications of hormones [77,78]; (2) effective antisera for immunohistochemistry are lacking; or (3) it is not certain whether endo- or pinocytosis rather than specific gene expression is responsible for a positive immunohistochemical result [79]. Thus, ISH has, for example, been used to detect neuroendocrine genes in small-cell lung carcinomas [80] and adrenocortical tumors [26]; calcitonin-related peptide and calcitonin in medullary thyroid carcinoma [81,82]; preproparathormone in hyperplastic, adenomatous, and carcinomatous human parathyroid glands [83]; and specific hormones in pituitary neoplasms [84] (Figs. 19 and 22).

In some instances, the combination of ISH and immunohistochemistry is used to localize peptides and its mRNA simultaneously in a single section [85,86] or alternatively in consecutive

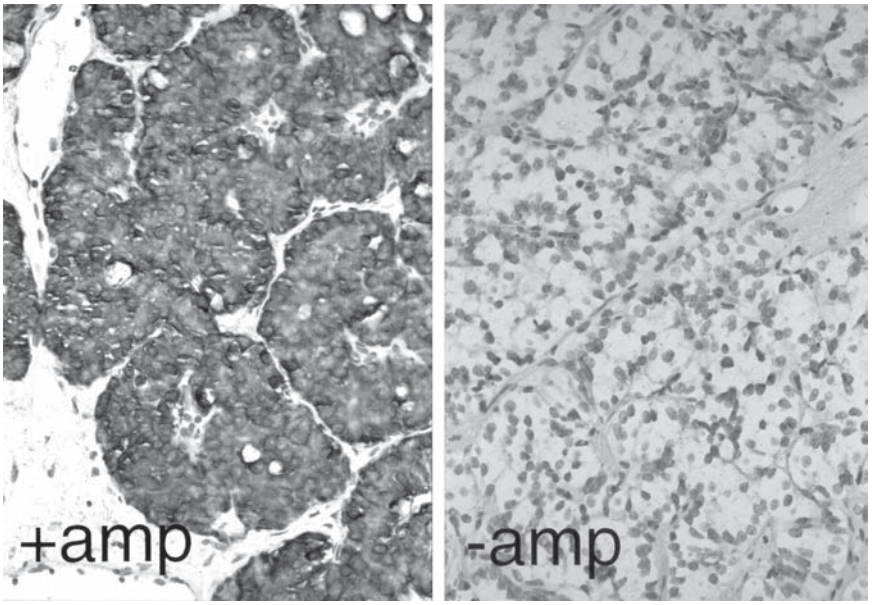


Figure 22 *In situ* detection of insulin mRNA in an insulinoma using a highly diluted, digoxigenin-labeled insulin antisense oligonucleotide probe followed by CARD amplification for visualization (**left**; +amp) and without amplification (–amp). Note the strong cytoplasmic signal after CARD amplification in contrast to the very weak signal without amplification.

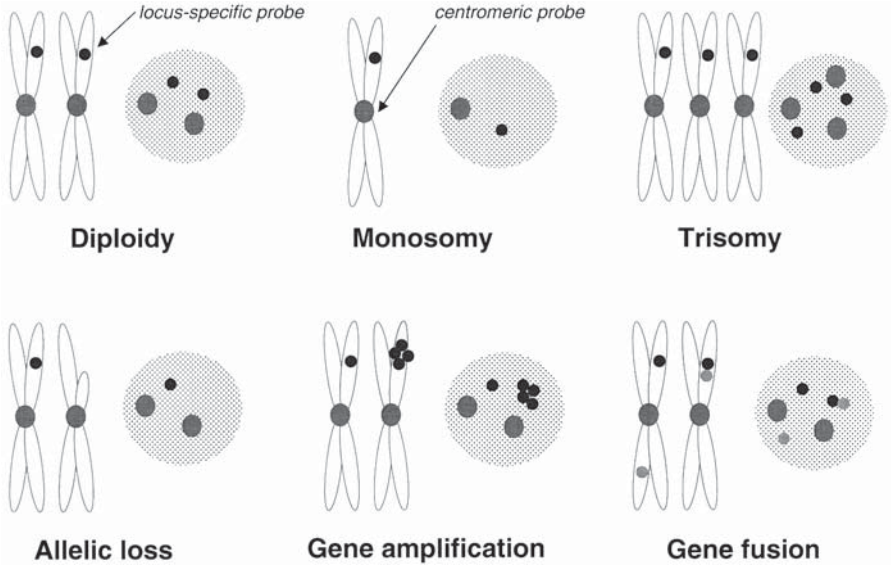


Figure 23 Schematic representation of FISH results using centromeric (chromosome-specific) and locus-specific probes comparing metaphase spreads (**left**) and interphase nuclei (**right**).

sections to provide evidence for the cell or tissue to be the site of synthesis [87–92]. For combined ISH and immunohistochemistry on the same slide, the ISH can be performed either as the first or the second step and in the latter case RNase degeneration has to be avoided using RNase inhibitors.

FISH Over the last decade, FISH has emerged as a powerful clinical and research tool for the assessment of target DNA dosages within interphase nuclei. Detectable alterations include aneusomies, deletions, gene amplifications, and translocations (Fig. 23), with primary advantages to the pathologist including its basis in morphology, its applicability to archival, formalin-fixed paraffin-embedded material, and its similarities to immunohistochemistry. Recent technical advances such as improved hybridization protocols, markedly expanded probe availability resulting from the human genome sequencing initiative, and the advent of high-throughput assays such as gene chip and tissue microarrays have greatly enhanced the applicability of FISH. With FISH, unique regions of the genome can be detected by applying complementary labeled nucleic acid probes. After denaturation of the DNA, the specific probes can bind (hybridize) to the target sequence on the chromosomes, forming a new DNA duplex. The bound probes are mostly visualized by fluo-

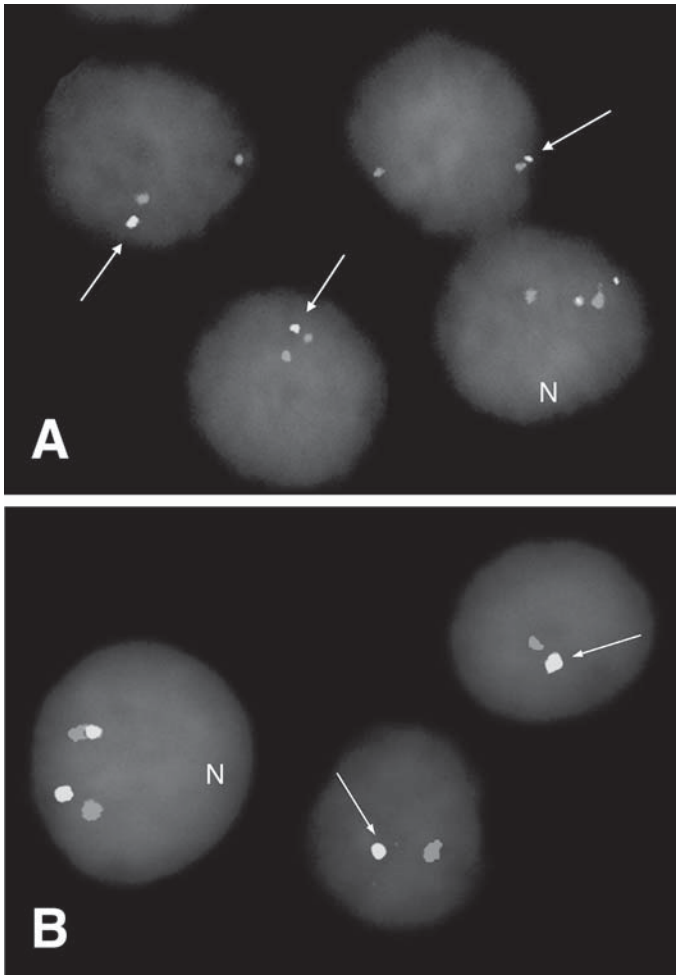


Figure 24 Double FISH in interphase nuclei of two endocrine pancreatic tumors (**A, B**) using a centromeric chromosome 11 probe (gray signal) and an *MEN1* specific probe (white signal). In the tumors cells of **A** only one white signal is present (*arrows*) but two gray signals are detectable (when compared to the normal cell, N), indicating a loss of one allele at 11q13. In the tumor cells of **B** one white and one gray signal is missing (when compared to the normal cell, N), indicating the loss of one chromosome 11.

Table 5
Selection of DNA Probes for the Detection of Chromosomes, Distinct Loci, or Genes Related to Endocrine Neoplasias by FISH Analysis

Gene/locus	*Related to*	*Reference*
X, Y chromosome anomalies	Pancreatic endocrine tumors	150
3p25.3–p23 loss	Pancreatic endocrine tumors	127
9q34 gain	Pancreatic endocrine tumors	151,152
6q22, 6q23–q24 loss	Pancreatic endocrine tumors	128
Chromosome 11	Multiple endocrine neoplasia type 1-associated pituitary adenoma	153
RET rearrangements	Thyroid papillary microcarcinoma	154
RET rearrangements	Childhood papillary thyroid carcinoma	155
Trisomies of chromosomes 7 and 12	Follicular thyroid carcinoma	156
Trisomies of chromosomes 5, 8, and 12	Prolactinoma	157
Chromosome 11	Pituitary adenomas	158
Mutant *RET* allele in trisomy 10	Multiple endocrine neoplasia type 2-associated pheochromocytomas	159

rescent dyes with either a direct or indirect detection method, and the results can be evaluated by fluorescence microscopy using appropriate filters (Fig. 24).

The probes are mainly generated using cloning vectors such as cosmids, plasmids, or P1 as well as yeast artificial chromosomes (YACs) and bacterial artificial chromosomes (BACs). In molecular cytogenetics, three different types of probes are generally used: probes specific for repeated sequences, whole chromosomes, or single-copy sequences. Among the first probes that have been applied in molecular cytogenetics are those directed against highly repetitive sequences in the centromeric regions of chromosomes. For all human chromosomes, cloned DNA probes against specific sequences in the centromeric region can be obtained commercially. Alternatively, highly specific DNA probes can be generated by primer-directed DNA in vitro amplification using PCR.

The major application of centromeric probes is assessing aneuploidy (e.g., monosomy, trisomy) in tumor cells by detecting gains or losses of whole chromosomes. In contrast to repeated sequences, unique sequences, for example, single-copy genes, are more difficult to visualize. Although FISH to single-copy sequences has been successfully performed with probes from cDNA clones that are shorter than 1 kb, cosmid-sized probes of approx 10–40 kb are more suited for those studies. Probes against single-copy genes can be used for identifying structural chromosome changes, that is, translocations, deletions, or gene amplifications (Fig. 23). So far, only a limited number of specific probes against single copy genes are commercially available.

In contrast to other human neoplasms, the FISH method is rarely applied for diagnostic purposes in endocrine pathology. Recent applications of FISH methods in the research of endocrine neoplasms are listed in Table 5.

LIQUID-BASED METHODS

Southern and Northern Blotting *Southern blotting* is a technique for the analysis of double-stranded DNA molecules [93] and *Northern blotting* for RNA molecules by filter hybridization. Both methods are infrequently used in diagnostic endocrine pathology and are occasionally applied for research purposes. Thus, for both techniques high-molecular-weight genomic DNA or total RNA of fresh tissue is required, which is rarely available for diagnostic purposes.

Prior to separation of DNA by electrophoresis to a solid-phase agarose gel, the DNA has to be digested with a restriction nuclease. The restriction enzyme cleaves the DNA at specific sites, where its recognition sequence occurs, resulting in the generation of DNA fragments that vary in size. In addition to the requirement of denaturation of the DNA, usually performed by alkaline treatment of the gel, it is necessary to partially hydro-

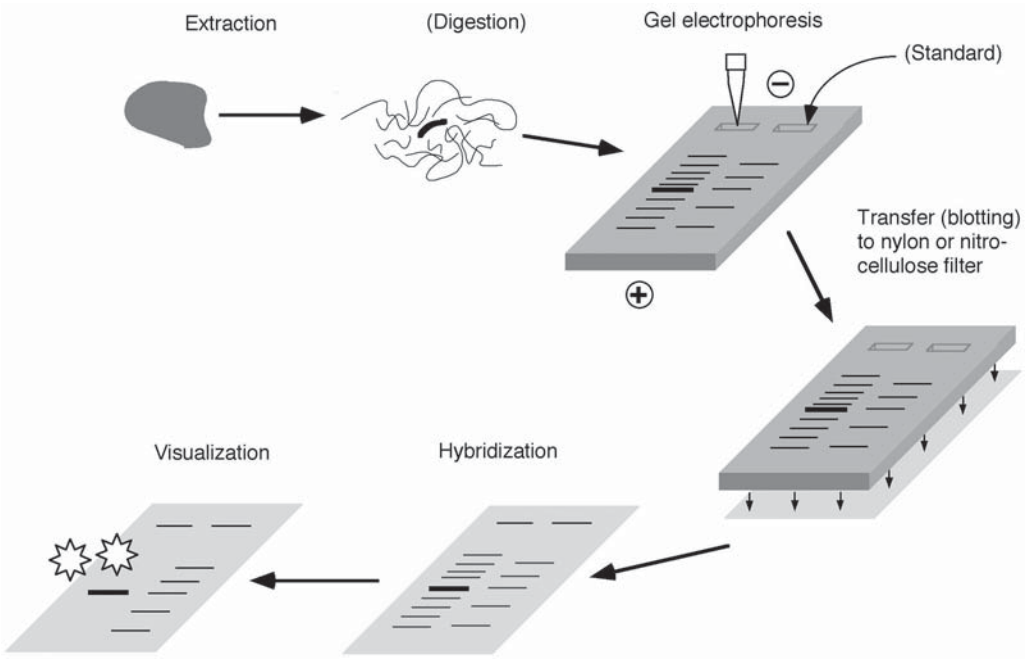


Figure 25 Principle of blot hybridization.

lyze the DNA to facilitate the transfer of larger DNA fragments (depurination in mild acid solution). The depurinated and denatured DNA fragments are transferred (blotted) to a nylon or nitrocellulose filter followed by detection of specific sequences with a radioactively or nonradioactively labeled probe that is capable of annealing to the DNA on the filter (see ISH) (Fig. 25). Applications of Southern blotting include the analysis of structural genetic changes (e.g., rearrangement, large deletion, or insertion of a gene), and specificity control of PCR products (using an adapted protocol) (Fig. 26).

Northern blotting is the RNA equivalent of Southern blotting. Because of the inherent differences between RNA and DNA, several modifications of protocols are required. Because RNA is single stranded, no denaturation is required and due to the relative small size of mRNA, no restriction enzyme digestion is needed. Owing to the complex secondary structure of RNA molecules, electrophoresis of RNA is carried out under denaturing conditions using formaldehyde (often included in the gel) and formamide (which is usually added to the RNA sample before loading for electrophoresis). It is not necessary to include a known molecular weight marker as in the electrophoresis of DNA. Samples contain ribosomal RNA, which yields predominant 28S and 18S bands equivalent to 4.7 and 1.6 kb, respectively. Analogous to Southern blotting, the RNA is transferred to nylon or nitrocellulose filters after electrophoresis and hybridized with a labeled DNA or antisense RNA probe. This technique allows measurement of the size as well as relative amount of mRNAs in a certain cell type or tissue. Furthermore, Northern blotting is useful to confirm the specificity of probes used for mRNA ISH.

Tissue Microdissection for DNA/RNA Extraction Tissues are complicated three-dimensional structures, composed of different types of interacting cell populations. Because the cell

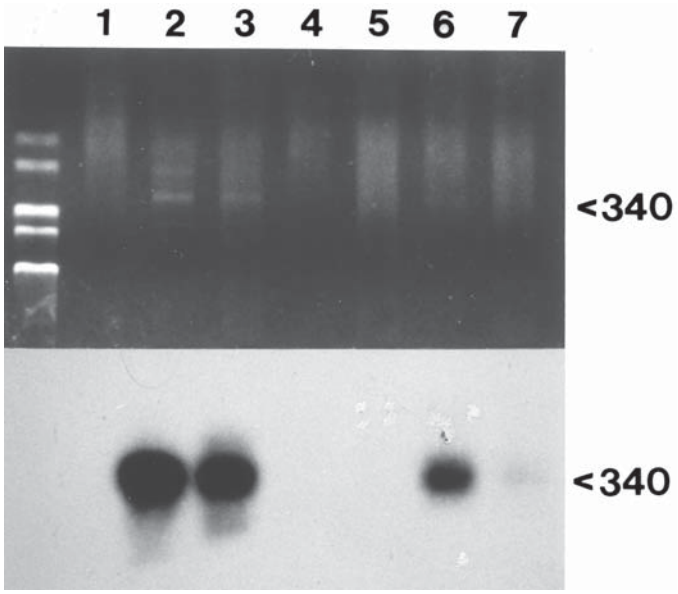


Figure 26 Example of Southern blot hybridization to confirm specificity of PCR products using a digoxigenin-labeled internal probe and chemoluminescence for visualization. Note that ethidium bromide staining of PCR products exhibits barely visible products of 340 base-pairs in *lanes 2, 3*, and *6* and that strong signals are detectable after Southern blotting (**lower panel**).

population of interest might constitute only a minute fraction of the total tissue volume, the problem of cellular heterogeneity has been a major barrier to the molecular genetic analysis of normal vs diseased tissue. Thus, tissue microdissection represents one of the most promising techniques in molecular pathology, offering a link between morphology and molecular genetic analysis.

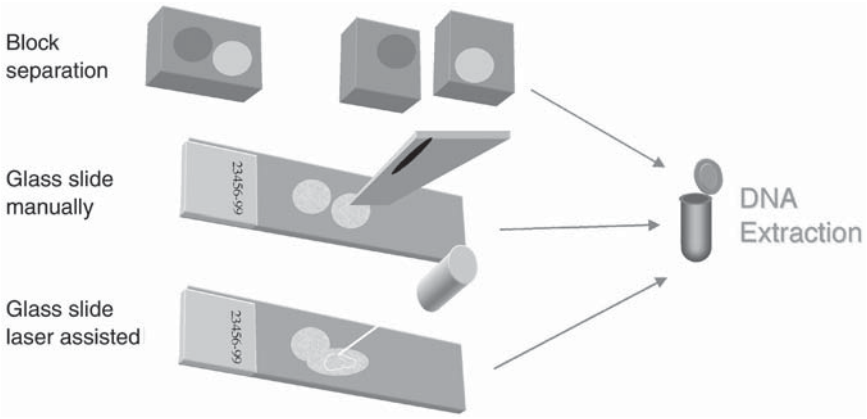


Figure 27 Schematic representation of tissue microdissection.

Table 6
Overview of the Most Common Microdissection Techniques in Molecular Pathology

	Manual tissue dissection	*Laser microbeam microdissection (LMM)*	*Laser pressure catapulting (LPC)*	*Microdissection of membrane mounted tissue (MOMeNT)*	*Laser capture microdissection (LCM)*
Function principle	Procurement of large tissue areas using a sterile needle or a scalpel with/without an inverted microscope	"Cold ablation" of unwanted cells using a UV laser (337 nm) Procurement of remaining cells with/without a micromanipulator under an inverted microscope	"Cold ablation" of unwanted cells using a UV laser (337 nm) Procurement with "noncontact" laser pressure catapulting	Polyethylene foil as supporting membrane allows to cut out single cells or cell groups	Melting effect between selected tissue and an transfer film due to local heating by a IR laser (980–1064 nm)
Minimum sample size	≅ 50–100 µm	< 1 µm	< 1 µm	1 µm	> 7 µm
Preferential spectrum of use	Large and homogeneous cell areas (>104 cells)	Small lesions (<50 cells), single cells (also suitable for chromosome microdissection)	Single cells	Single cells or small cell groups (<50 cells)	Small cell groups (5–20 cells), large single cells
Sample procurement	Manual (sterile needle, scalpel) Micromanipulator	Computer-assisted micromanipulator	"Noncontact" laser pressure catapulting directly into the sample tube	"Noncontact" laser pressure catapulting directly into the sample tube	Thermoplastic transfer film

Tissue microdissection can be applied to routine tissue sections of both *paraffin-embedded* and *frozen tissue* as well as to *cytological preparations*. It enables the isolation of morphologically well-defined cells or cell groups that can be further processed for molecular genetic analysis. Microscopic control allows the definition of malignant or even premalignant cells and their dissection from the surrounding non-neoplastic tissue. The dissectates represent purified pools of morphologically well-defined cells with no or minimal contamination by non-neoplastic cells.

Principles of Tissue Microdissection Precision, avoidance of contamination, and efficiency of the procedure are the most important parameters in tissue microdissection. The spectrum of techniques ranges from paraffin block dissection to manual microdissection and single-cell preparation based on laser- and computer-assisted systems (Fig. 27). An overview of the most common microdissection techniques in molecular pathology is given in Table 6. In general, the isolation of premalignant or malignant lesions by microdissection requires a well preserved histo- or cytomorphology and a trained pathologist.

Manual tissue dissection can be performed on routinely stained slides using 5–15-µm-thick sections placed on noncoated glass slides. Manual tissue dissection requires histologically homogeneous malignant lesions, and the areas should have a

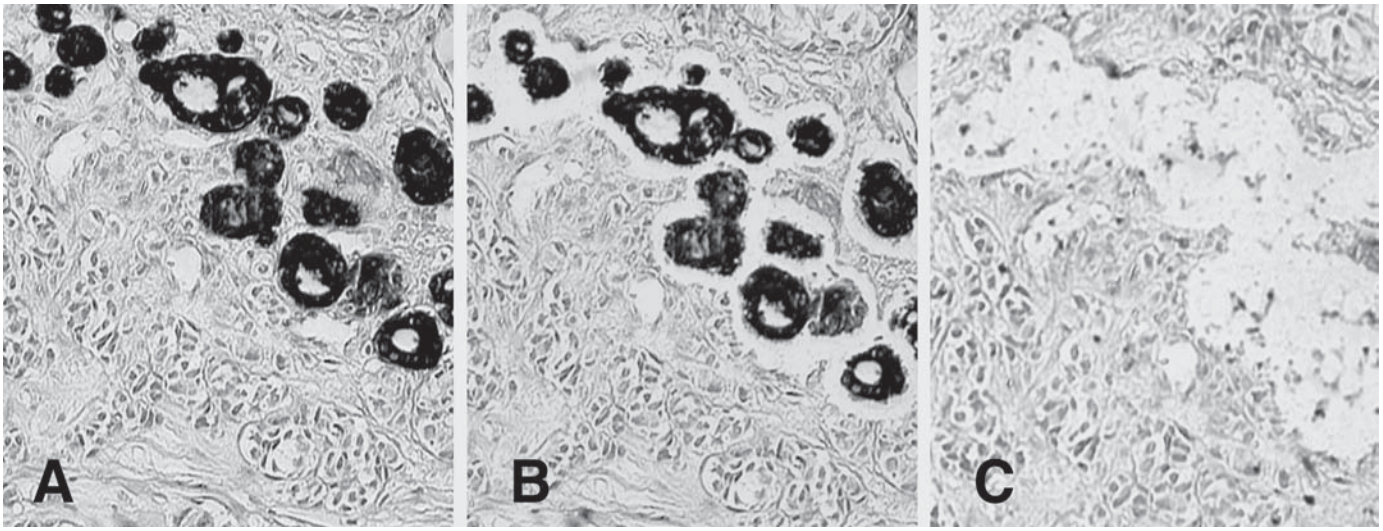


Figure 28 Laser-assisted microdissection of thyroglobulin stained follicles (**A**) in a mixed medullary–follicular thyroid carcinoma. Follicles are isolated by laser (**B**) and catapulted into a Eppendorf tube for molecular analysis. (**C**) Tissue section after removal of follicles.

diameter of at least 1 mm [94]. Using a sterile needle or a scalpel, the selected lesions can be procured [95].

The principle of *laser cutting* is a locally restricted ablative photodecomposition process without heating the direct environment of the laser beam [96]. Within the diffraction-limited focus of the laser beam obtained by a high numerical microscope lens, a very-high-energy density is available, and if the pulse duration is shorter than the relaxation time of the biological material (range of microseconds), heat transfer is avoided [97]. In this way a pulsed UV laser microbeam can be used to cut or ablate stromal, inflammatory, or residual parenchymal cells surrounding the tumor cells in histological sections without destruction of genetic information of the remaining cells as shown with different experiments [98]. At the site of laser exposure and ablation, no amplifiable material is left behind [99]. To retrieve the cells from the slide, usually a computer-controlled micromanipulator or conventional sterile needles are used to pick and transfer the cells in a tube for further molecular analysis.

Laser pressure catapulting (LPC) allows one to catapult an isolated cell or cell group out of its surrounding with a single precisely aimed laser shot [100]. The ejected dissectates are either caught on a small piece of cover glass or are directly catapulted onto the cap of a common PCR tube. The greatest advantage of this method is the procurement of the material in a "noncontact" manner, which minimizes the risk of contamination.

For microdissection of membrane-mounted native tissue (MOMeNT) the tissue sections are mounted onto a 1–3 μm polyethylene foil (P.A.L.M., Bernried, Germany), which is attached to a slide with nail polish [101]. With an UV laser microbeam, tissue areas can be cut out with high precision. Combining this method with LPC, one single laser shot makes it possible to catapult cell groups or even whole tissue areas of up to 1000 μm in diameter (Fig. 28). However, this method is more suited to procure small cell groups and single cells, if no or only minimal contamination by non-neoplastic cells is wanted [102]. The MOMeNT technique requires a special slide preparation with polyethylene foils, and excludes the use of routinely processed glass slides.

Laser capture microdissection (LCM) is helpful to select and procure cell clusters from tissue sections by use of a laser pulse. In LCM, a thermoplastic polymer coating attached to a rigid support is placed in contact with a tissue section. The polymer over microscopically selected cell clusters is precisely activated by a near-infrared laser pulse, and then bonds to the targeted area. Removal of the polymer and its support from the tissue section procures the selected cell aggregates for molecular analysis. Once the cells are captured, the DNA, RNA, or protein can be easily extracted from the isolated cells. The spectrum of applications of this technique is wide, and it allows the fast procurement of histologically homogeneous tissue areas or single cells [103]. A great advantage is the well-preserved morphology of the transferred cells, which are attached to the removed polymer and can be readily visualized under the microscope. Ease and rapidity of the use has been achieved by the commercial LCM microscope (Arcturus Engineering, Mountain View, CA, USA; http://www.arctur.com). However, the focal spot of the melting laser cannot reach below 7 μm in diameter and there is no possibility to destroy selectively unwanted cells or tissue, neither adjacent nor within the selected area.

Tissue Sources Formalin-fixed and paraffin-embedded biopsies provide the main source of tissue for molecular analysis. Routine sections (5 μm) stained with hematoxylin and eosin (H&E) are commonly used for tissue microdissection. Other histologic stains such as methyl green or nuclear fast red may also be used [104]. The sections can be mounted on routine glass slides for most techniques of microdissection. Immunohistochemical staining of the tissue sections prior to microdissection offers an additional phenotypic characterization [102,105]. It is helpful to increase the histo- and cytomorphology by covering the stained sections with a thin layer of xylene or 2-propa-

nol, which improves by wetting and refractive-index matching the morphology on the unmounted slides. The xylene or 2-propanol evaporates quickly before cell procurement.

Sections from fresh frozen tissue can also be used for tissue microdissection [106,107]. For an immunophenotypical characterization immunohistochemical staining procedures can also be applied to frozen sections. However, the exact assessment of histomorphological details may be hampered in frozen sections.

The examination of cytological preparations from several organs such as the uterine cervix is well established for identifying premalignant or malignant cells. Routinely prepared cell smears stained with Papanicolaou stain can be used for microdissection and subsequent PCR analysis, even after storage of several years [108]. Other cell preparations, for example, cytospin samples, are also suitable to isolate cells or cell groups by microdissection.

DNA Extraction from Microdissectates From the microdissected cells DNA isolation according to standard procedures is possible, if the samples contain at least 10^5 cells. However, the dissectates most often represent smaller samples. Thus, a simple one-step DNA preparation is recommended [99]. The resulting DNA preparation is not "clean," but is sufficient for PCR-based analysis.

PCR-directed amplifications require a careful control of reaction parameters, such as quality and quantity of the DNA template, to ensure reliable results. In contrast to the analysis of DNA that has been extracted from tissue specimens without dissection, an accurate quantitation of template DNA obtained by microdissection before PCR analysis has so far been made difficult because of low amounts of DNA available for measurement. Although the amount of DNA extracted from microdissected cells seemingly can be estimated by counting the absolute number of dissected cells, significant deviations from the expected results may occur. It is obvious that all investigations aimed at the absolute quantitation of target sequences present within microdissected cells require a precise quantitation of the template DNA as an exclusive precondition.

RNA Extraction from Microdissectates RNA from microdissected tissue can be obtained by standard methods using commercially available RNA isolation kits. Microdissection by UV laser-based techniques must be carefully performed to eliminate all bystander cells because high-copy mRNA transcripts from contaminating cells can produce erroneous results. Precipitating fixatives, such as ethanol and acetone, are believed to produce more reverse transcriptase PCR (RT-PCR) amplification products than crosslinking fixatives such as formaldehyde (109). However, we found no differences in the qualitative expression of several genes in formalin-fixed compared to fresh-frozen tissue [110]. For fewer than 10^5 cells, RNA amplification techniques should be applied.

PCR The PCR technique is now so pervasive in molecular biology that it is difficult to think of life without it. Innovative researchers have continually updated the definition of "PCR applications," increasing the usefulness and scope of the technique.

Principles of PCR PCR is an in vitro method for enzymatically synthesizing defined sequences of DNA. The reaction uses two oligonucleotide primers that hybridize to the opposite strands and flank the target DNA sequence that is to be amplified. The elongation of the primers is catalyzed by a heat-stable DNA polymerase (such as *Taq* DNA polymerase). A repetitive series of cycles involving template denaturation, primer annealing, and extension of the annealed primers by the polymerase results in exponential accumulation of a specific DNA fragment. The ends of the fragment are defined by the 5' ends of the primers. Because the primer extension products synthesized in a given cycle can serve as a template in the next cycle, the number of target DNA copies approximately doubles every cycle. Thus, 20 cycles of PCR yield about a million copies (2^{20}) of the target DNA (Figs. 29 and 30).

Quantitative determination of DNA sequences and gene expression levels offers a powerful approach for the comparative analysis of normal and diseased specimens, especially in neoplastic endocrine tissues. Remarkable progress has been made in recent years in the development of techniques for assessing DNA copy number and gene expression at the mRNA level. For instance, *real-time PCR* allows the exact quantification of DNA or RNA (RT-PCR) in tissue. More recently, microarray analysis techniques have been developed for quantitative large-scale analysis of gene copy numbers or gene expression. However, a crucial factor for the reliability of the results obtained with these advanced techniques is the use of morphologically well defined cell populations.

Genomic PCR Alterations in gene copy numbers are one of the most important causes of deregulated gene expression and neoplastic transformation. Investigations of the pathogenic or prognostic significance of gene amplification require a reliable, sensitive, and objective method for the determination of gene copy numbers in tumor samples (Fig. 31). The recent introduction of fluorescence-based kinetic PCR procedures offers a new tool for a very sensitive and accurate quantification of even minute amounts of nucleic acids (Fig. 32). In principle a quantitative real-time PCR assay can be developed and validated for all loci in the human genome for which sequence information is available.

Degenerate Oligonucleotide Primer (DOP)-PCR If only a limited amount of DNA template is available, amplifying the DNA sample uniformly may make later manipulations (e.g., cloning, labeling, and hybridization reactions) more efficient. DOP-PCR provides a universal method for uniform amplification of small DNA samples [111]. The DOP-PCR procedure is useful as a first step in ISH with flow-sorted chromosomes, comparative genome hybridization (CGH), or preparation of size-fractionated DNA fragments (e.g., for subtractive hybridization). Primers used for DOP-PCR have defined sequences at the 5' end and at the 3' end, but have a random hexamer sequence between the two defined ends. The random hexamer sequence displays all possible combinations of the natural nucleotides A, G, C, and T. DOP-PCR primers are annealed at low-stringency to the denatured template DNA and hybridize statistically to primer binding sites. The distance between primer binding sites can be controlled by the length of the defined sequence at the 3' end and the stringency of the annealing conditions. The first five cycles of the DOP-PCR thermal cycle consist of low stringency annealing, followed by a slow temperature increase to the elongation temperature, and primer elongation.

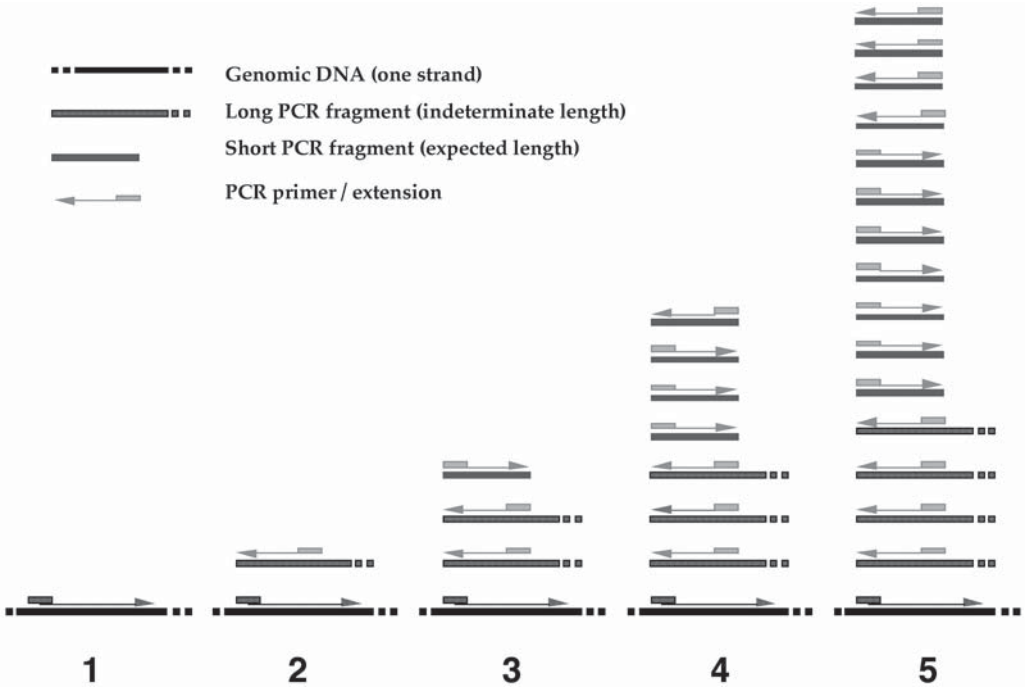


Figure 29 Principles of PCR (one DNA strand). Note the linear amplification of PCR products of intermediate length (flanked by one primer) and the exponential increase of numbers of PCR products of expected length (flanked by both primers) after the second cycle.

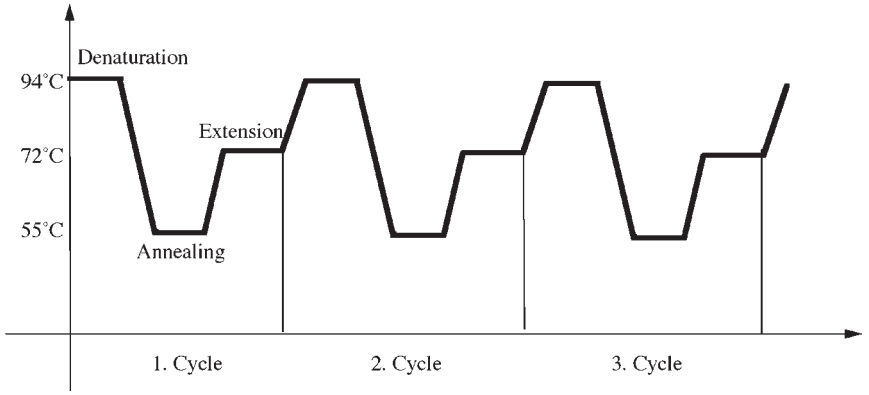


Figure 30 Schematic representation of temperature cycling during PCR to yield DNA denaturation, primer annealing, and primer extension.

The next 35 cycles use a more stringent (higher) annealing temperature. Under the more stringent conditions, the material that was generated in the first five cycles is amplified preferentially, as the complete primer sequence created at the amplicon termini is required for annealing. DOP-PCR amplification ideally results in a smear of DNA fragments that are visible on an agarose gel stained with ethidium bromide.

RT-PCR Determination of mRNA levels of specific genes is becoming increasingly important as a measurement of gene expression. With the recent advent of RT-PCR, the sensitivity for mRNA determination has been increased dramatically, and this technique is becoming widely used in studies that involve small tissue samples and/or isolated cells. The possibility to measure PCR product accumulation during the exponential phase of the reaction using fluorescent data has revolutionized not only DNA but also RNA quantitation. PCR and RT-PCR permit the quantitative determination of minimal starting quantities of nucleic acids down to at least 500 copies of a target sequence and are therefore particularly suited as downstream applications in combination with microdissection. Very recently, it was demonstrated that quantitative RT-PCR can also be applied to study gene expression in microdissected tissue samples from archival formalin-fixed tissues (Fig. 32) [109,112]. Specht et al. [112] assessed the influence of several RNA extraction techniques, formalin fixation, and laser-assisted microdissection on mRNA quantitation. They demonstrated that expression level determinations from archival tissues were comparable to matched frozen specimens when using small target sequences in a range of 60–100 bp for real-time RT-PCR amplification.

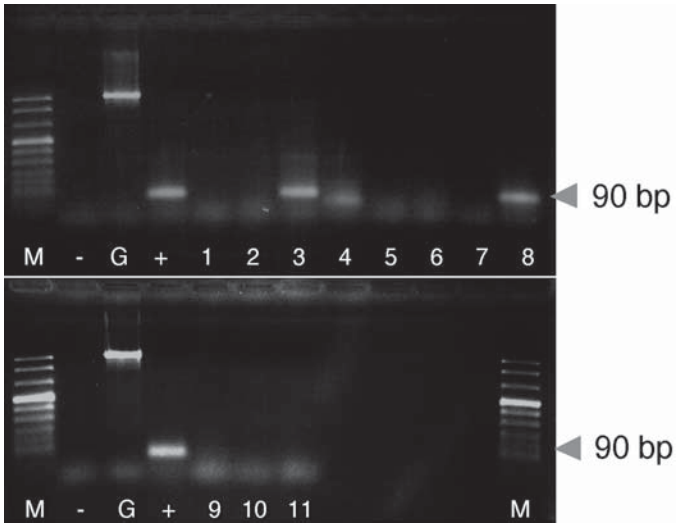


Figure 31 Detection of RET mRNA expression in archival papillary thyroid carcinoma samples using RT-PCR. Note the positive signals in *lanes 3, 4,* and *8*. –, Negative control; G, genomic control DANN; +, positive control.

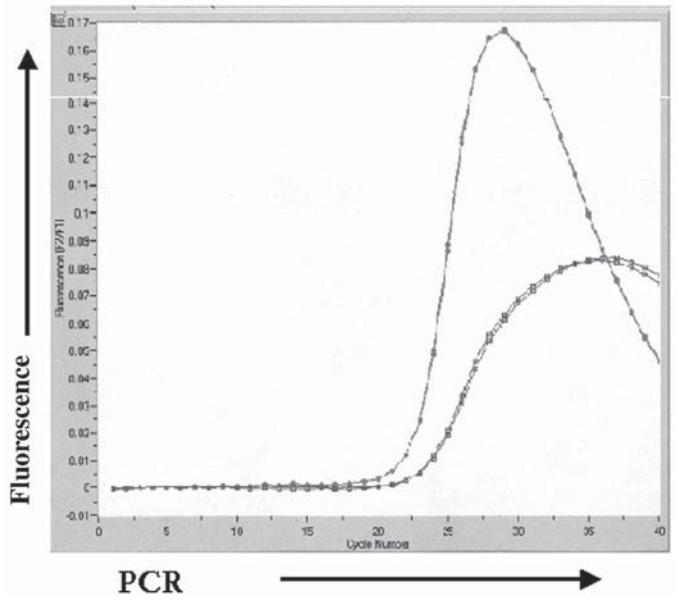


Figure 32 "Real time" RT-PCR analysis of an archival tissue sample. The graph represents the detection of a reference and a marker gene (each in duplicate) via flourescence monitoring of PCR products. During the early phases of the reaction (PCR cycle 0–18) no products are detectable (no fluoresence signals), whereas specific flourescence signals, beginning at PCR cycle 21 and 23 onwards, indicate the presence of gene-specifc PCR products.

Thus, mRNA recovery and quantitative analysis is possible even from archival routine microdissected specimens. This suggests that these tissues can serve as a useful template for real-time RT-PCR analysis of a broad range of individual genes as well as newly developing high-throughput gene expression methodologies. The RT-PCR method is rarely used in diagnostics but more frequently applied for research purposes. Recent applications of quantitative RT-PCR in endocrine neoplasms are listed in Table 7.

Table 7
Selection of Recent Applications of Quantitative RT-PCR to Endocrine Tumors

Gene expression	*Related to*	*Reference*
Cyclooxygenase-2	Thyroid nodules	110
$K\alpha_1$-tubulin	Thyroid anaplastic carcinoma	160
MYC, *ERBB2*, and *CCND1*	Malignant thyroid follicular cell tumors	161
Adrenomedullin, leptin, their receptors, and neuropeptide Y	Hormone-secreting and nonfunctioning pituitary adenomas	162
Menin	Various adrenal tumors	163
Telomerase activity	Primary cultures of normal adrenocortical cells	164
Calcium-sensing receptor	Gastrinomas	165
Renin–angiotensin system	Pancreatic endocrine tumors	166

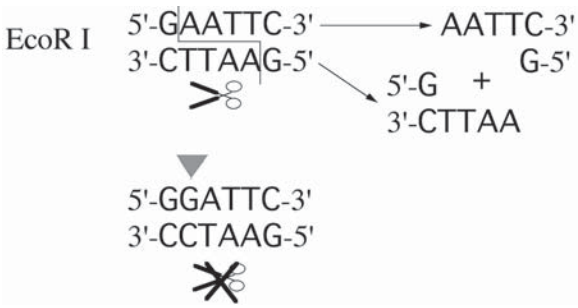


Figure 33 Schematic representation of the *Eco*RI restriction site. Loss of the restriction site occurs by an A → G point mutation (*arrowhead*).

Mutation Analysis PCR-based methods are favored today for mutation analysis because they are easy to perform and require only small amounts of DNA. Different approaches to mutation analysis are needed in different situations. Screening methods such as single-strand conformation polymorphism (SSCP) or denaturing gradient gel electrophoresis (DGGE) are able to indicate a sequence difference compared to the wild-type DNA. However, these methods cannot discriminate between polymorphisms and mutations and do not indicate the exact nucleotide exchange. For defining the precise nucleotide sequence of an unknown mutant DNA strand, direct sequencing is the method of choice. Once a mutation has been defined by direct sequencing, in a family, for example, further mutation analysis can be done using restriction enzyme digestion with a restriction enzyme that cuts only the mutant DNA strand (Fig. 33).

DNA Sequencing Today *direct sequencing* is performed according to the dideoxy chain termination method described by Sanger [113]. A DNA polymerase produces a complementary strand to the matrix DNA, which can be obtained by PCR, for example. In four separated reactions, dideoxy forms of the four nucleotides are added to the usual deoxynucleotides. The dideoxynucleotide is integrated into the newly synthesized DNA strand and leads to a specific termination of DNA synthesis because the 3' hydroxyl group is missing for the attachment

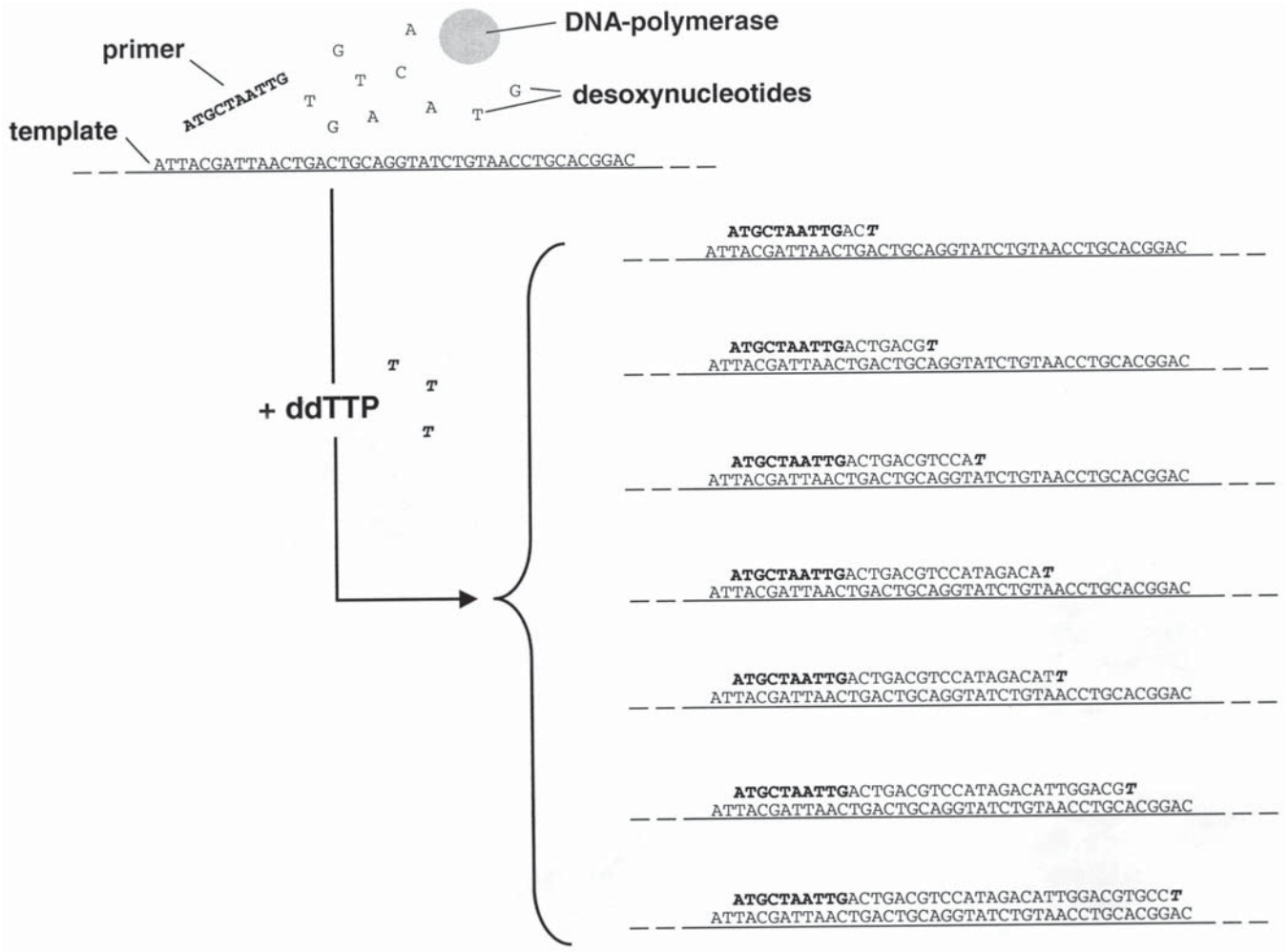


Figure 34 Principle of DNA sequencing using the dideoxy chain termination method. The addition of, for example, dideoxy thymidine triphosphate nucleotides (ddTTP) to the reaction mixture leads to newly synthesized fragments of varying size that end with thymidine. By separation of the products of the four different reactions (using ddATP, ddTTP, ddCTP, ddGTP) in a gel electrophoresis, the sequence can be read from bottom to top of the gel.

of the following nucleotide. Owing to the mixture of normal and dideoxy nucleotides, fragments of varying size are produced during the reaction. They start with the primer sequence and end, for example, with guanine, if dideoxyguanine is added to the reaction (Fig. 34). The products of the four reactions are run separately on a denaturing polyacrylamide gel and the sequence of the synthesized strand, complementary to the sample strand, can be read from the bottom to the top of the gel. To visualize the fragments, either the primer or the nucleotides are labeled (^{35}S or ^{32}P). Using different fluorophores for the four dideoxy nucleotides, all four reaction products can be loaded into the same line. The output is in the form of intensity profiles for each of the differently colored dideoxynucleotides and is stored electronically. This automated sequencing made large-scale sequencing possible [114] (Fig. 35).

Single-Strand Conformation Polymorphism (SSCP) The SSCP method is an efficient screening method for genetic alterations [115]. The PCR products are denatured by heating and

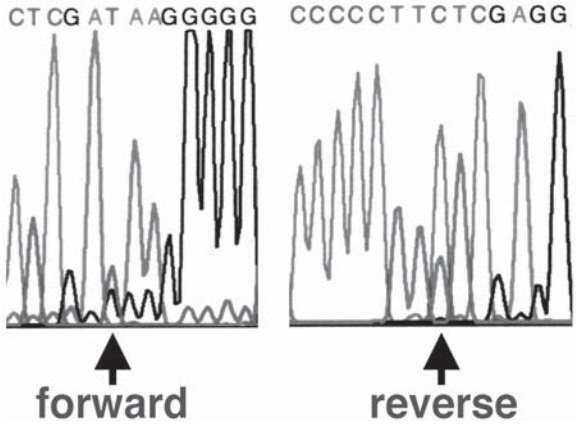


Figure 35 Example of sequencing results using automated nonradioactive cycle sequencing with four different fluorochromes and intensity profiles. Note the double peaks in the forward and reverse sequence (*arrows*), indicating a heterozygous point mutation.

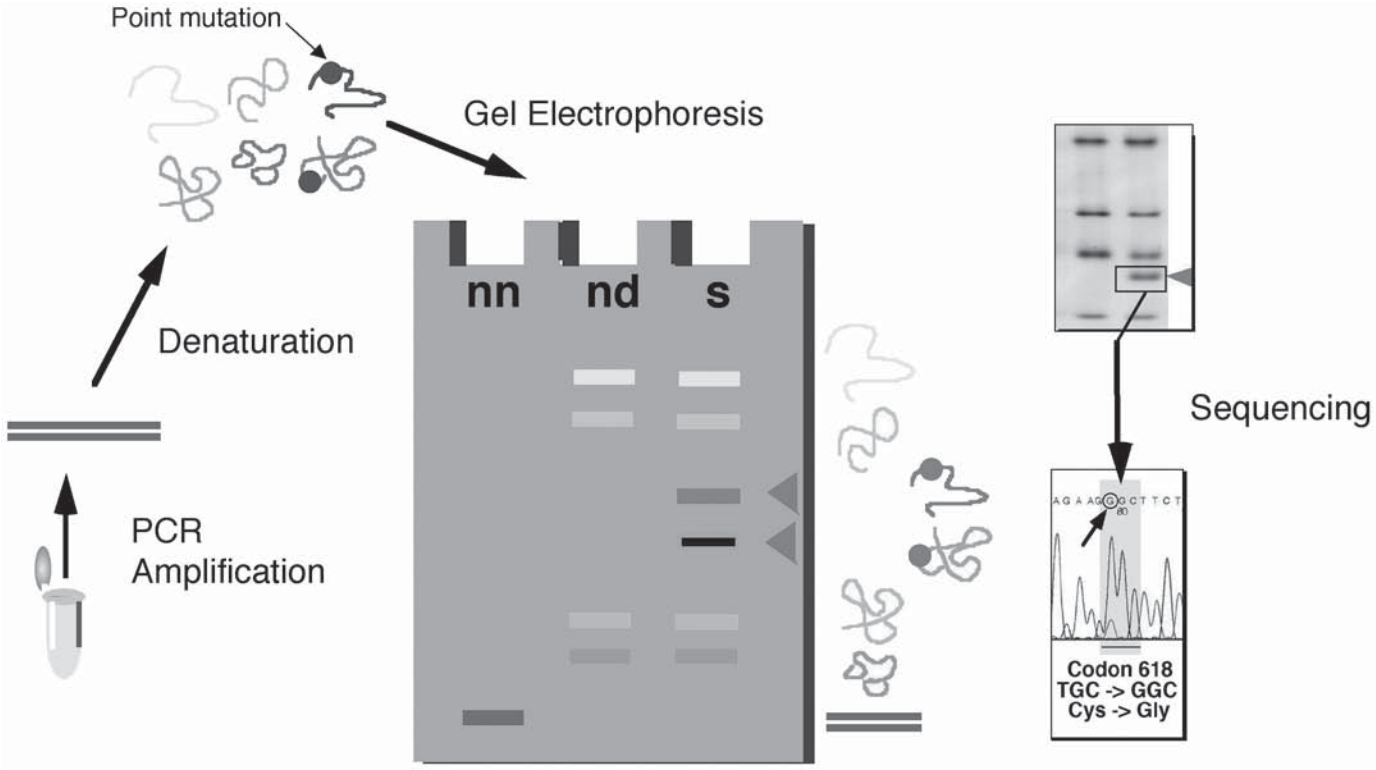


Figure 36 Principles of single-strand conformation polymorphism (SSCP) analysis. A point mutation leads to band shifts in the gel electrophoresis (*arrowheads*). nn, Normal DNA nondenatured; nd, normal denatured; s, sample DNA.

then electrophoresed on a nondenaturing polyacrylamide gel. The single-stranded DNA molecules form three-dimensional structures according to their primary nucleotide sequence. Small alterations of the sequence including point mutations can lead to changes in the three-dimensional structure of DNA strands. The DNA is then visualized by autoradiography or silver [116, 117] or ethidium bromide staining. Wild-type DNA leads to two bands corresponding to the forward and reverse strands. Nucleotide substitutions lead to a differential banding pattern. The banding pattern must always be compared to the pattern of the wild-type sequence. The additional bands can be cut out of the gel, and subsequently reamplified and sequenced. SSCP is estimated to detect 70–95% of mutations in a fragment of 200 bp; the sensitivity decreases with increasing size of the probe. The principle of this assay is shown in Fig. 36.

Denaturing Gradient Gel Electrophoresis (DGGE) The DGGE method also relies on the different mobilities of wild-type and mutant DNA [118]. The target sequences are chosen so as to have a homogeneous melting temperature throughout using computer software (Win-melt). A so-called GC clamp, a region rich in guanine and cytosine residues 20–60 bp long, is added on the 5' end of one primer to generate a steep increase in the melting temperature. The PCR products are run on a gel containing a gradient of DNA denaturing agents such as urea and formamide in a given temperature (60°C). The moment the products attain their melting point the DNA strands denature, remaining attached only in the region of the GC clamp. In this state the migration is retarded. The presence of nucleotide substitutions results in a different melting temperature of the products and hence in a different banding pattern. The DNA bands are again visualized by silver or ethidium bromide staining. Once the primers and conditions are established, this method detects virtually 100% of mutations in fragments up to 800 bp long. Again, samples leading to a differential banding pattern compared to the wild-type alleles are directly sequenced. The principle of DGGE is shown in Fig. 37.

Applications of Mutation Analysis in Endocrine Pathology To date, the main application of mutation analysis in endocrine pathology is the discrimination between sporadic and familial tumor forms. In patients with a suspicious phenotype or family history, blood DNA is screened for the mutation of the suspected gene. It is important to know that the absence of a mutation in a tumor suppressor gene does not rule out a given syndrome, as there could still be a germline deletion of a whole allele that is not detected by mutation analysis. Germline deletions, however, are an infrequent finding [119,120]. Mutation analysis is used, for example, for the identification of disease gene carriers of multiple endocrine neoplasia type 1 (MEN 1), MEN 2 [121,122], and other familial diseases (Table 8); in patients with a suspicious phenotype; and, more important, in their family members to detect disease carrier status (Fig. 38). By this means, cumbersome lifelong biochemical screenings can be avoided in unaffected persons, whereas disease gene carriers can be screened more thoroughly or treated prophylactically (e.g., prophylactic thyroidectomy in MEN 2 carriers). Mutation analysis in sporadic tumors is—thus far— used only for research purposes as yet no somatic mutations with strong clinical impact have been identified [123].

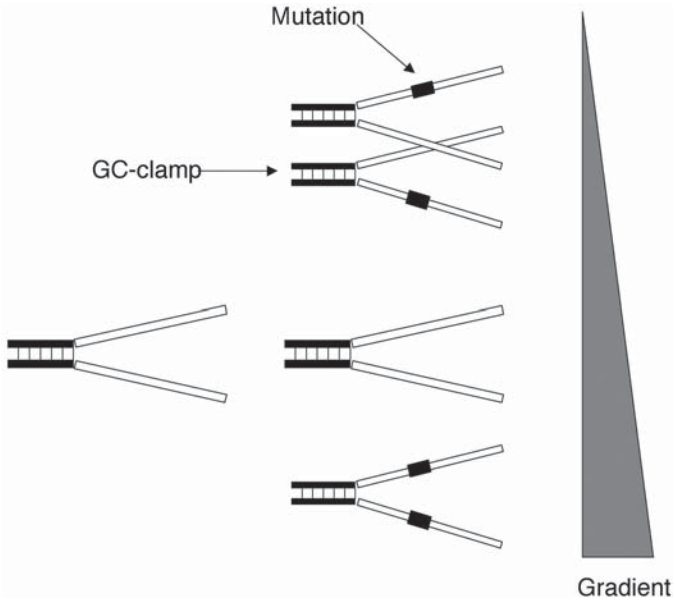


Figure 37 Principles of denaturing gradient gel electrophoresis (DGGE). Separation of PCR products with a point mutation (*right lane*) in a gradient gel leads to additional bands with altered migration when compared to PCR products of wild-type DNA (*left lane*). The *upper* two fragments represent so-called heterodimers (one mutated and one wild-type strand) and the lower two so-called homodimers (two identical strands per product).

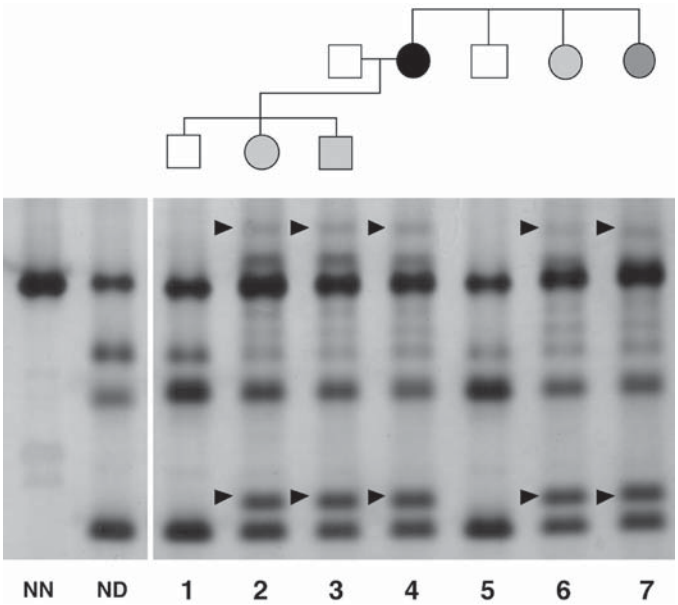


Figure 38 Mutation screening using SSCP analysis of RET exon 10 in a MEN 2 family. Note that not only the index patient with a medullary thyroid carcinoma (*black circle*) but also two of her children and two sisters exhibit pathological band shifts, indicating a disease gene carrier status.

Table 8
Familial Syndromes with Endocrine Tumors

Disease	*Locus*	*Gene*	*Type*
Multiple endocrine neoplasia type 1 (MEN 1)	11q13	*MENIN*	S
Multiple endocrine neoplasia type 2 (MEN 2)	10q11.2	*RET*	O
von Hippel–Lindau (VHL)	3p25.5		S
Neurofibromatosis type 1 von Recklinghausen (NF 1)	17q11.2	*Neurofibromin*	S
Familial paragangliomas	11q23	*SDHD*	S
	1p36.1–35	*SDHB*	
	1q21	*SDHC*	
McCune–Albright	20q13.2	*GNAS1*	O
Carney complex	17q22–24	*PRKAR1a*	S
	2p16		

SDH, Succinate dehydrogenase complex; S, suppressorgene; O, oncogene.

Loss of Heterozygosity (LOH) Analysis Using Microsatellite Markers According to the Knudson hypothesis [124] inactivation of a tumor suppressor gene occurs in two steps. The first mutation is a point mutation or some other small genetic change confined to one allele of the tumor suppressor gene. This mutation might be a germline (leading to an inherited disease such as MEN) or somatic (leading to a sporadic tumor mutation). The second mutation is often a large genomic loss of a part of a chromosome or even a whole chromosome. This LOH frequently leads to loss of polymorphic markers flanking the tumor suppressor gene [125]. Microsatellites are tandem repeats of simple sequence (usually 1–4 bp) that occur abundantly and at random throughout the human genome. Trinucleotide and tetranucleotide tandem repeats are often highly polymorphic and can thus be used as polymorphic markers. Because they are usually <100 bp long and are flanked by DNA with unique sequences, they can be amplified in vitro using the PCR (Fig. 39). Such polymorphic markers flanking the tumor suppressor gene are used for LOH analysis. It is of advantage to choose markers with a high rate of heterozygosity in the population to minimize the number of uninformative cases. If the markers are applied to paraffin-embedded material, attention should also be paid to product size, as it is difficult to amplify large DNA fragments from formalin-fixed tissues. PCR with these primers is then performed on "pure" tumor tissue and in parallel non-neoplastic tissue of the same patient. The PCR products are then electrophoresed on denaturing gels and visualized by autoradiography or by silver staining. Usually a ladder of (PCR products) bands is observed rather than two discrete bands resulting from the maternal and paternal allele. This "stutter" is due to addition of adenosine nucleotides to the PCR products by the *Taq* polymerase. If the normal tissue shows two distinct bands, this marker is suited to distinguish the two alleles of the patient and can be used to examine the tumor for allelic loss. If the normal tissue leads to one single band, the patient is homozygous for this polymorphic marker and thus the result will not be informative. If the PCR products of the tumor tissue show only one product in patients with two products in their normal tissue, allelic loss occurred at the examined marker (Fig. 40). Interpretation is more difficult in tumors with admixtures of non-neoplastic tissue such as inflammatory cells or vessels. In these

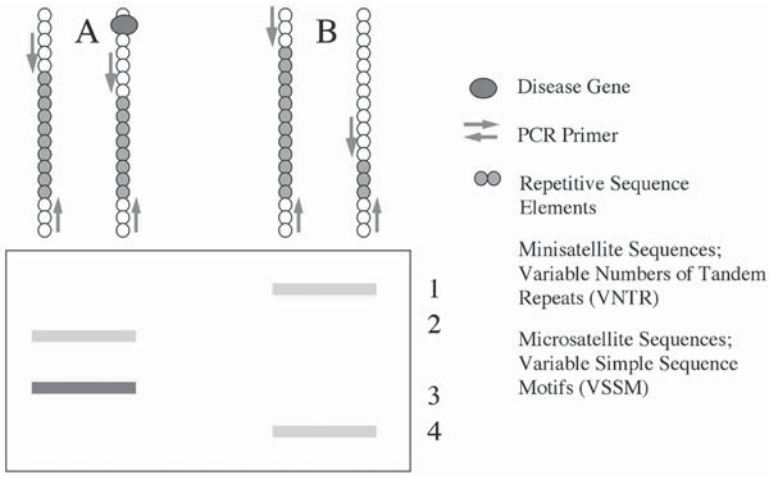


Figure 39 Schematic representation of microsatellite analysis using PCR. Primers flanking a polymorphic region are used to amplify the maternal and paternal alleles of a chromosomal region (note the four different alleles identified in two different individuals). Loss of one allele in tumor tissue is indicative of loss of heterozygosity of a tumor suppressor gene in the vicinity of the polymorphic region.

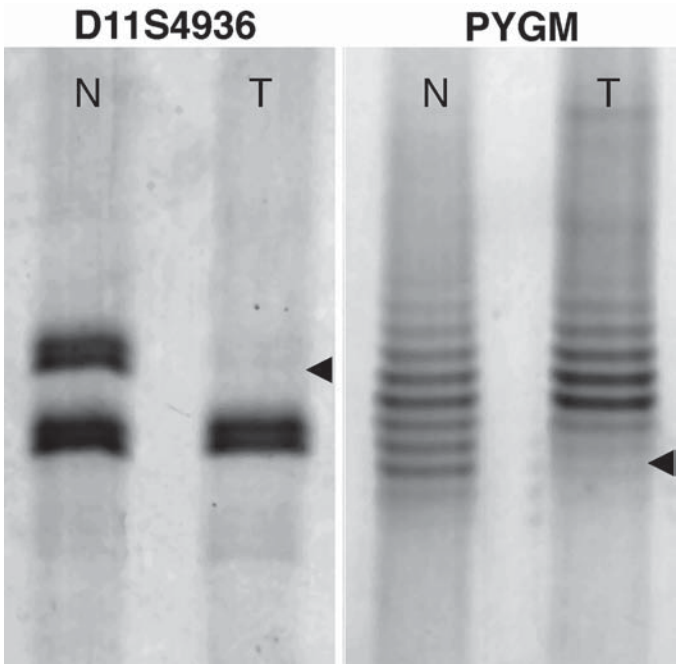


Figure 40 Example of allelic loss of chromosome 11q in an endocrine pancreatic tumor using two different polymorphic markers (D11S4936; PYGM). Note the lost bands (*arrowheads*) in the tumor DNA (T) when compared to the DNA of nontumorous tissue of the same individual (N).

cases a 50% reduction of band intensity is required to diagnose LOH and careful microdissection of tumor tissue is crucial to minimize "contaminating" non-neoplastic tissue. The use of fluorescent primers in combination with analysis of PCR products in fluorescent DNA sequencers using specialized software (Genscan) makes a quantification of the results possible. LOH is defined as ratios >1.5 for loss of the shortest allele or <0.5 for the largest [126].

Instead of showing loss of one allele a few tumors may show new alleles in the tumor DNA. This is due to defects in the DNA repair machinery of the tumor cells. Whereas this phenomenon of microsatellite instability or replication error (MSI/RER) is seen in about 13% of colorectal, gastric, and endometrial carcinomas, it seems to be very rare in endocrine tumors.

Applications of LOH Analysis in Endocrine Pathology The LOH method is rarely used for diagnostic purposes but frequently applied in research to screen tumors for allelic losses and thus to identify genomic loci harboring possible tumor suppressor genes. In endocrine pancreatic tumors, for example, genetic regions on 3p and 6q could be identified [127,128]. These regions are more frequently lost in metastasizing endocrine pancreatic tumors and thus seem to harbor tumor suppressor genes important for malignant behavior of these tumors. Other studies have demonstrated a variety of allelic losses in different endocrine tumors [116,129–131] and those results may help to identify tumor suppressor genes that are involved in the neoplastic transformation and progression of these neoplasms.

Comparative Genomic Hybridization (CGH) Although FISH has substantially improved metaphase chromosome classification, its application in solid tumors is still limited by the difficulty of interpreting the complex karyotypes. CGH, developed in 1992, partially overcomes this by mapping changes in relative DNA sequence copy numbers onto normal metaphase chromosomes.

Principles of CGH In CGH, total genome DNAs from tumor and reference samples are labeled independently with different fluorochromes or haptens and cohybridized to normal chromosome preparations along with excess unlabeled Cot-1 DNA to inhibit hybridization of labeled repeated sequences. The ratio of the amounts of the two genomes that hybridize to each location on the target chromosomes is an indication of the relative copy number of the two DNA samples at that point in the genome. The remarkable level of genomic abnormality is apparent (Figs. 41 and 42).

The principal advantages of CGH are as follows: (1) it maps changes in copy number throughout a complex genome onto a normal reference genome so the aberrations can be easily related

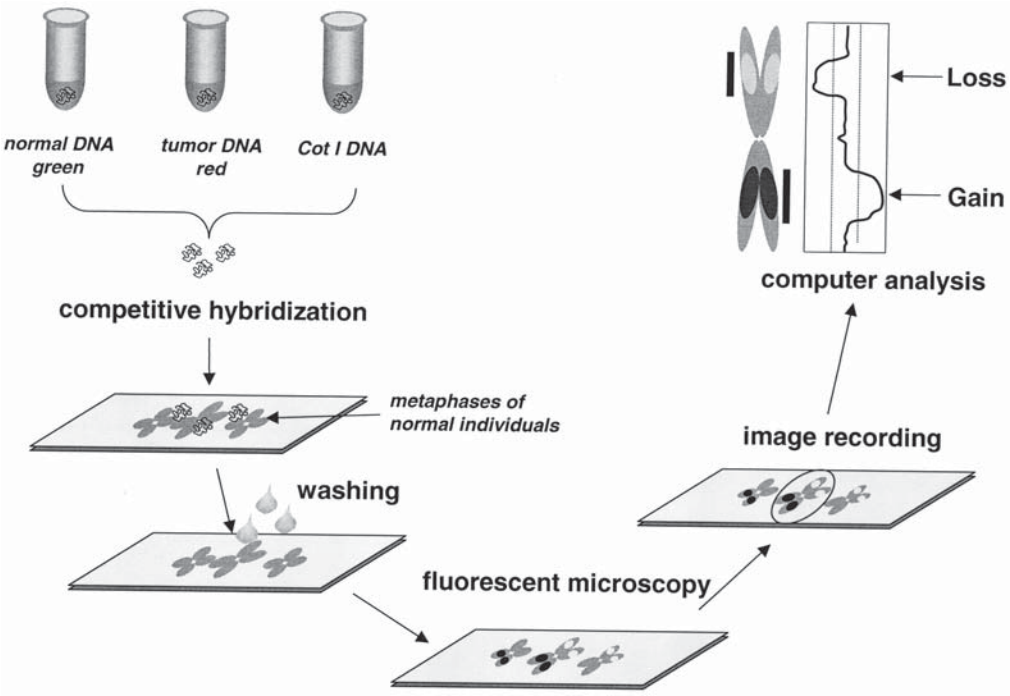


Figure 41 Principles of comparative genomic hybridization (CGH).

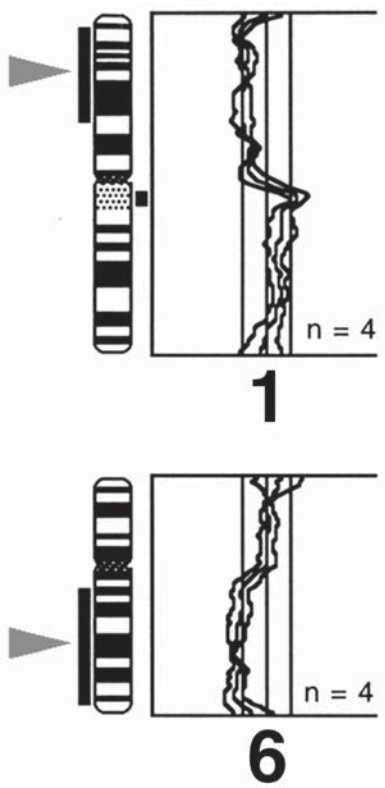


Figure 42 Example of a CGH result in an endocrine pancreatic tumor. The *arrowheads* mark losses of DNA on the short (p) arm of chromosome 1 and on the long (q) arm of chromosome 6.

to existing physical maps, genes, and genomic DNA sequences; and (2) it employs genomic DNA so that cell culturing is not required. The latter point is especially helpful in endocrine pathology, as many neuroendocrine tumors are difficult to cultivate. The main limitations of chromosome-based CGH are: (1) it is limited in resolution to 10–20 Mb, (2) it does not provide quantitative information about gene dosage, and (3) it is insensitive to structural aberrations that do not result in a DNA sequence copy number change.

Replacing metaphase chromosomes as the substrate onto which aberrations are mapped with arrays of well mapped cloned nucleic acid sequences can eliminate some of these limitations. The arrays are constructed using a robot to place clone DNA in high-density arrays on glass substrates. Array densities as high as 104/cm can now be achieved. Initial work involved CGH to arrays comprised of targets spanning >100 kb of genomic sequence such as BACs [132]. More recently, STS-mapped YAC clones were used as targets achieving more than doubled coverage of the chromosomal region of interest (Fig. 43). In completion to common cDNA array studies, this approach appears to be useful and clearly demonstrates that changes in genome copy number can be detected and mapped at a resolution defined by the genomic spacing of the clones used to form the array. Furthermore, CGH matrix array allows quantitative assessment of DNA sequence dosage from one copy per test genome to hundreds of copies per genome [132]. The high resolution of the CGH matrix array compared with the chromosome CGH and the opportunities for quantitative aberration definition are apparent in genomic analysis, as the approach of microarray CGH has now been demonstrated in several laboratories [132–134].

Very recently, matrix CGH has been demonstrated [135] on adrenocortical tumors. Zhao et al. [135] used a microarray-based CGH technique, combined with conventional CGH, to identify

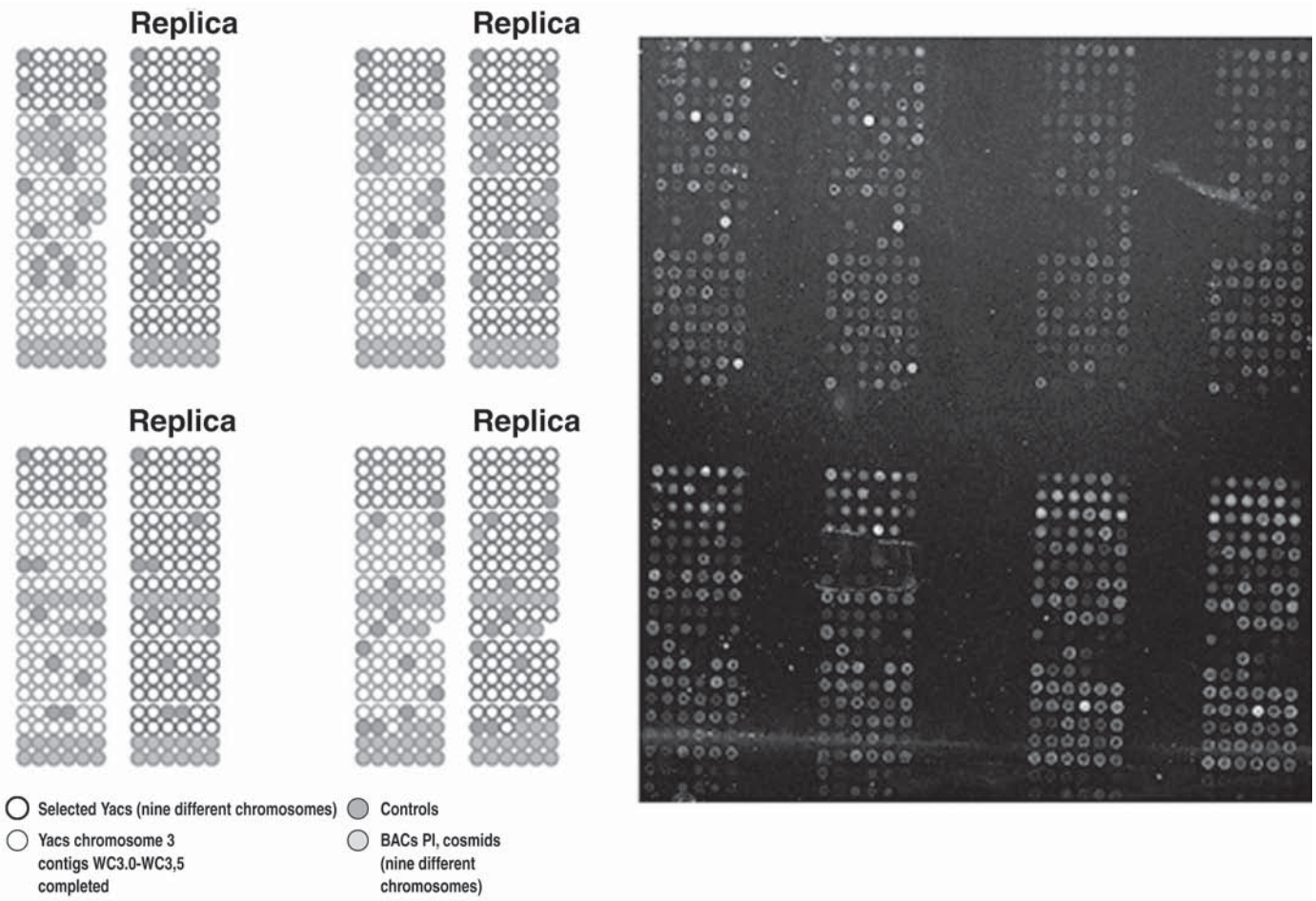


Figure 43 Example of a CGH microarray experiment. (**Left**) CGH microarray design (chromosome 3): 479 DNA probes and 479 replicas; 373 different ALU-PCR products from YAC clones. In addition, 54 available BAC, P1, and cosmids were spotted on a glass slide. Five different 96-well plates of template DNA probes were used for this particular microarray design. Controls (52 probes of normal DNA) were dispersed randomly over the entire microarray. (**Right**) Composite image of a differential hybridization generated using two different fluorescent dyes Cy3 (false color green) for labeling of tumor DNA and Cy5 (false color red) for normal control DNA. The genomic DNA used for hybridization was obtained from microdissectates from approx 10,000 cells.

gene amplifications in 35 adrenocortical tumors. Using microarrays, they demonstrated coamplifications of *SAS/CDK4* and *MDM2* in adrenocortical tumors. cDNA arrays are attractive for CGH because they are increasingly available and carry a very large number of clones. However, the sensitivity of cDNA clone-based CGH for detection of low-level copy number changes is likely to be less than that for CGH matrix arrays based on YAC or BAC clone DNA.

CGH is used mainly for research purposes not only in endocrine pathology but also other organ systems. Recent applications of CGH in endocrine neoplasms are listed in Table 9.

Array Technology

cDNA Microarrays The concept of expression profiling led to the development of robotic methods for arraying thousands of cDNAs on microarrays. These cDNA arrays that can be spotted on either nylon filters or glass slides are hybridized with labeled aRNA or cDNA to generate a molecular fingerprint of a specific cell type, disease state, or therapeutic efficacy (Fig. 44). The highly parallel data acquisition and data analysis on cDNA arrays allows the exact determination of complex changes in gene expression. Apart from cDNA microarrays, several other methods have been devised to study gene expression on a large scale: cDNA subtraction, differential display, representational difference analysis (RDA), expressed sequence tag (EST) sequencing, serial analysis of gene expression (SAGE), and differential hybridization on either high-density spotted nylon filters or glass. Profiles of gene expression obtained with all these techniques, however, are reliable and meaningful only, if they can be assigned to morphologically identified pure cell populations. Until recently, the application of cDNA array techniques has been limited to mRNA isolated from millions or, at very best, several thousand cells, thereby restricting the study of small samples and complex tissues. Because the total RNA content of mammalian cells is in the range of 20–40 pg and mRNA accounts for only 1–5% of the cellular RNA, any attempt at single-cell profiling must be capable of dealing with a total of 105–106 mRNA molecules. Nonamplified RNA from microdissected tissue samples has been used as a radioactive probe for cDNA arrays; however, at least 5000–50,000 microdissected cells are required for this type of analysis [136,137].

Table 9
Selection of Chromosomal Changes Detected by CGH in Endocrine Tumors

Involved chromsomes/chromosomal regions	*Related to*	*Reference*
Gain of 1q and loss of 9q21.3–q32	Prognosis in papillary thyroid carcinoma	167
Chromsomes 19q, 19p, 13q, and 11q	Medullary thyroid carcinomas	168
Chromosomes 5 and 8	Anaplastic thyroid carcinoma	169
Loss on 16p	Anaplastic thyroid carcinoma	170
Gain of chromosomes 7, 5 , 9, 12, 14, 17, 18, and X	Benign and malignant follicular thyroid tumors	171
Gains of chromosomes X, 19, 12, 7, and 9 Loss of chromosomes 11, 13, and 10	Sporadic pituitary tumors	172
3p25.3–p23 loss	Pancreatic endocrine tumors	127
9q34 gain	Pancreatic endocrine tumors	151,152
6q22, 6q23–q24 loss	Pancreatic endocrine tumors	128
Gain on 4q, 5q (5q13 → 5q23), 9p (9p21 → 9pter), 13q (13q21 → 13q32), 17q	Nonfunctioning pituitary tumors	173
Loss of chromosome 11q	Paragangliomas	174
Loss of chromosomes 1p and 3q	Sporadic pheochromocytomas (as early genetic events)	175
Gains high-level amplifications on 1p34.3-pter, 1q22–q25, 3p24-pter, 3q29, 7p11.2–p14, 9q34, 11q12–11q13, 12q13, 12q24.3, 13q34, 14q11.2–q12, 14q32, 16p, 17q24–q25, 19p13.3, 19q13.4, and 22q11.2–q12	Adrenocortical carcinoma	176

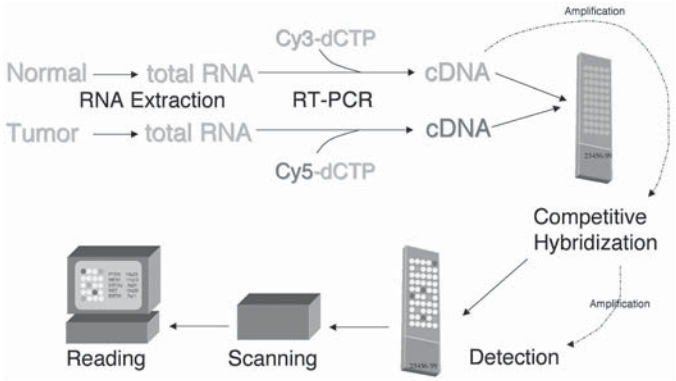


Figure 44 Schematic representation of the expression array method.

Table 10
Recent Applications of cDNA Microarrays in Endocrine Pathology

Selection of differentialy expressed genes detected by cDNA microarray	*Related to*	*Reference*
MET, LGALS3, KRT19, DPP4, MDK, TIMP1, FN1,CITED1, CHI3L1, ODZ1, N33, SFTPB, and *SCEL*	Papillary thyroid carcinoma	177
Folate receptor gene, ornithine decarboxylase gene, C-mer protooncogene tyrosine kinase gene	Pituitary adenomas	178
Matrix CGH	Mouse islet carcinomas	179

An array is an orderly arrangement of samples. It provides a medium for matching known and unknown DNA samples based on basepairing rules and automating the process of identifying the unknowns. An array experiment can make use of common assay systems such as microplates or standard blotting membranes, and can be created by hand or make use of robotics to deposit the sample. In general, arrays are described as macroarrays or microarrays, the difference being the size of the sample spots. *Macroarrays* contain sample spot sizes of about 300 μm or larger and can be easily imaged by existing gel and blot scanners. The sample spot sizes in *microarrays* are typically <200 μm in diameter and these arrays usually contain thousands of spots. Microarrays require specialized robotics and imaging equipment that generally are not commercially available as a complete system.

DNA microarray or DNA chips are fabricated by high-speed robotics, generally on glass but sometimes on nylon substrates, for which probes with known identity are used to determine complementary binding, thus allowing massively parallel gene expression and gene discovery studies.

Thus far, expression arrays are mainly applied to research. However, it is anticipated that with the help of automated systems specialized "prognostic" and "therapeutic" arrays will be available in the near future, which will allow for a tailor-made therapy of the affected patient. Recent applications of expression arrays in the research of endocrine neoplasms are listed in Table 10.

Tissue Microarrays The implementations of high-throughput genetic technologies, such as oligonucleotide microarrays, generate myriad points of data. The identified potential candidate genes need to be characterized further and selected using a large number of well-characterized tumors and stringent criteria. Tissue microarrays allow for such high-throughput expression profiling of tumor samples, providing, in addition, information

at the microanatomical level. Different techniques could be applied for identification of specific phenotypic (immunohistochemistry and ISH) or genotypic (FISH) alterations, holding strong potential for translational research. Tissue microarrays consisting of 0.6-mm biopsies of paraffin-embedded tissues are well validated and have been used for various clinicopathological studies.

Tissue microarrays carrying three cores of paraffin-embedded tumors per specimen deliver accurate results and allow economic high-throughput processing of cancer specimens and other tissues. This technology has the potential to accelerate translational research and to analyze efficiently tissue expression of genes identified by DNA microarray studies. In addition, multi-cell-line arrays are useful for rapid characterization of the expression profiles of multiple cell lines relevant for cancer research. Both tissue and cell line arrays are powerful tools for the screening of new reagents like hybridization probes and antibodies. The standardization of staining procedures and reduction of intraassay variability can also be significantly improved with this technique. Tissue microarrays are useful for establishing large disease-specific tissue collections for future analysis of new targets in a particular tumor entity and can be helpful for collaborations between major institutions. The new tissue microarray techniques can be used for various different array designs such as (1) progression arrays containing precursor lesions of cancer and cancer specimens of lesions with increasing aggressiveness; (2) tissue-type comparative arrays containing normal, benign, and malignant specimens from the same tissue type; or (3) cancer arrays containing different subtypes or stages of the same tumor entity.

Clonality Analysis Assessment of clonality is an important factor in differentiating reactive from neoplastic lesions. In lymphomas or soft tissue sarcomas, clonality can be assessed by the identification of associated genetic changes such as clonal rearrangements of the immunoglobulin heavy chain or T-cell receptor or translocations. As in endocrine tumors no such general genetic lesions are known, a more general approach is needed.

Principles of Clonality Analysis Female cells carry a double amount of X-chromosomal genes. To avoid an increased expression of these genes either the maternally or paternally derived X chromosome is inactivated. This inactivation occurs during embryogenesis by random methylation of one X chromosome in each cell [138,139]. As this process is stable during subsequent cell divisions [140], normal tissues in adult females are cellular mosaics differing in which of the two X chromosomes is methylated. In contrast, neoplasms derived from a single somatic cell show a uniform pattern of X-chromosome inactivation indicating cellular monoclonality (Fig. 45).

Different activities of the X-chromosomal glucose-6-phosphate dehydrogenase (G6PD) isoenzymes were the first approach to analyze clonality using X-inactivation patterns [141]. However, the low frequency of heterozygosity (2%) for the G6PD isoenzymes made this approach impractical. Methylation-sensitive restriction enzymes, which selectively cleave nonmethylated DNA, allow discrimination between the active (unmethylated) and inactive (methylated) X chromosome on DNA level. First Southern blotting of the restriction fragment length polymorphisms (RFLPs) in the phosphoglycerate-kinase 1 gene (*PGK-1*) and hypoxanthine phosphoribosyltransferase (*HPRT*) gene was applied [142]. The variable repeat sequence in the *Dx255* locus identified by the M27 β probe made clonality analysis more practicable because of a high heterozygosity rate of approx 90%, but still all these Southern blotting approaches required high quantities of DNA and hence fresh tissue. The identification of a trinucleotide repeat (CAGn) in exon 1 of the androgen receptor gene (*HUMARA*) [143] with nearby methylation-sensitive restriction endonuclease sites for *Hpa*II and *Hha*I made a PCR-based approach possible (Fig. 46). These methylation-sensitive enzymes cut the DNA strand only if their recognition sequence is not methylated. Approximately 90% of females are heterozygous with respect to the number of CAG repeats in this region. After digestion with one of these restriction enzymes, PCR yields only products of the methylated, inactive allele of the androgen receptor. The included CAG repeat results in different product size of the two alleles. The PCR products are run on a denaturing polyacrylamide gel and are visualized by auto-

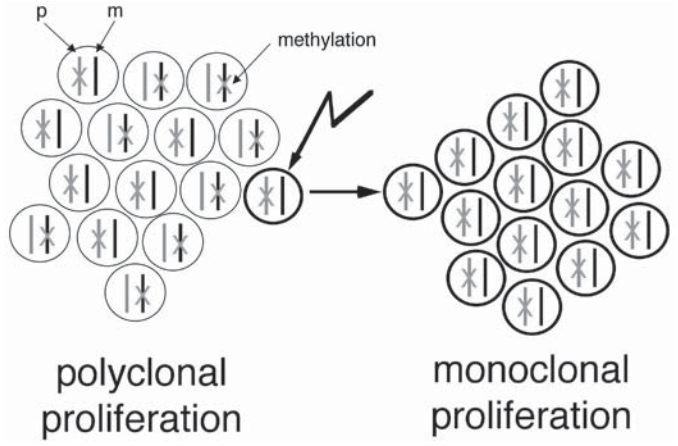


Figure 45 Schematic representation of X-chromosomal inactivation and clonality in a neoplasm of a female patient. Normal tissue in adult females is a cellular mosaic differing in which of the X-chromosomes is methylated (polyclonal proliferation). In contrast, monoclonal proliferations are derived from a single somatic cell and thus show a uniform pattern of X-chromosomal inactivation. p, Paternal allele; m, maternal allele.

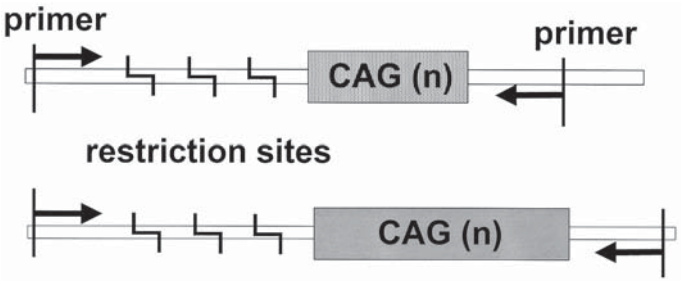


Figure 46 Exon 1 of the androgen receptor gene. A highly polymorphic short tandem repeat sequence (CAGn) close to the methylation-sensitive restriction sites *Hha*I or *Hpa*II allows for a specific amplification of the methylated (inactive) allele using restriction enzyme digestion prior to PCR.

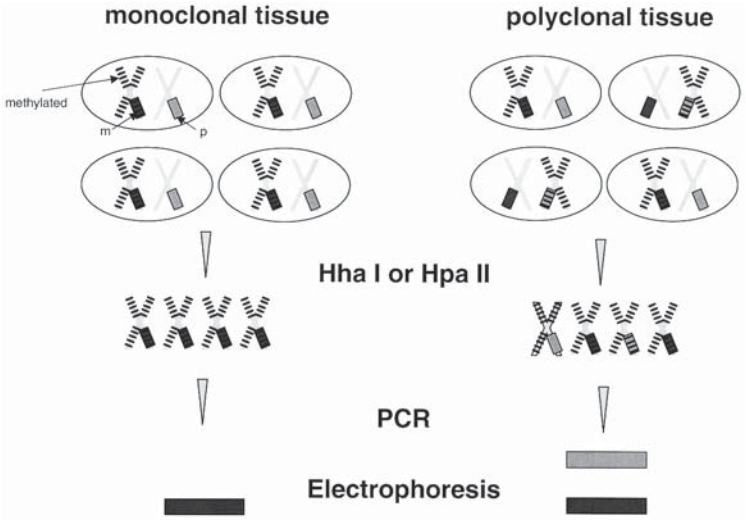


Figure 47 Schematic representation of clonality analysis using the HUMARA assay. The active allele of the X-chromosome is selectively cleaved by restriction analysis prior to PCR amplification of the polymorphic short tandem repeat. Disappearance of one band when compared to normal tissue of the same individual indicates monoclonality. m, Maternal allele; p, paternal allele.

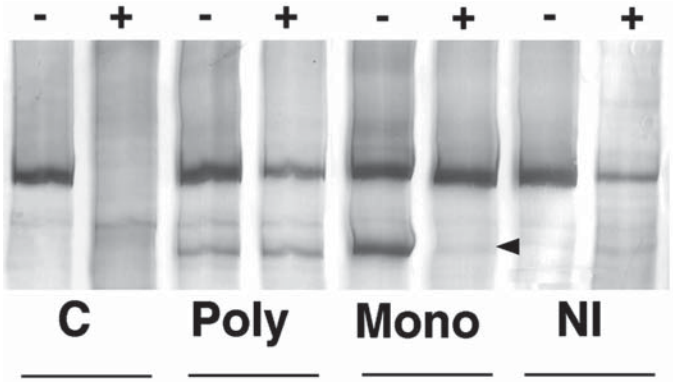


Figure 48 Example of clonality analysis. –, No digestion; +, PCR products after digestion; C, control DNA (male); Poly, polyclonal tissue; Mono, monoclonal tissue; NI, not informative (both alleles have the same length of CAG repeat).

radiography or by silver staining (Fig. 47). As for LOH analysis, the use of fluorescent primers permits quantitative analysis on automated sequencers. The critical step of this assay is the restriction enzyme digestion and appropriate controls (DNA of a male patient and tissue with known monoclonality) must be included in each run (Fig. 48).

Methylation-sensitive PCR [144] is a different approach that is not dependent on methylation-sensitive restriction digestion. Bisulfite treatment converts unmethylated but not methylated cytosine residues to uracil. PCR amplification is then performed with two sets of primers specific either for the unmethylated or for the methylated alleles.

RT-PCR approaches of polymorphic X-chromosomal genes avoid the difficult distinction between methylated and unmethylated DNA by directly examining mRNA expression [145].

Applications of Clonality Analysis to Endocrine Pathology

Clonality analysis in endocrine tumors has led to intriguing results, as histologically hyperplastic processes [146] can be monoclonal and neoplastic processes can be polyclonal [95]. Therefore, it seems today that the definition of a neoplasm cannot solely rely on monoclonality [146–149].

FUTURE ASPECTS

It is anticipated that with the techniques available at present and the information provided by the completed Human Genome Project, the molecular basis of many endocrine neoplasms will be elucidated in the near future. In consequence, our daily routine work as pathologists may look somewhat different in a few years. It may include the application of new assays on slides or extracted tissues, of additional immunohistochemical and molecular markers, as well as the necessity to use adapted classifications of tumors based on additional criteria derived from molecular studies.

In the meantime we should remain involved in the progress of research by ensuring that only tissues of well characterized tumors of patients with comprehensive follow-up data are included in ongoing studies to achieve the goal of understanding the molecular aspects of endocrine neoplasms.

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2 Hypothalamus and Neurohypophysis

KAZUHIRO TAKAHASHI, MD,
OSAMU MURAKAMI, MD, AND TORAICHI MOURI, MD

A BRIEF HISTORICAL OVERVIEW

An English scientist, Geoffrey Harris, first suggested that the brain controlled the pituitary gland through chemical mediators [1]. He supposed that the cells of the hypothalamus might synthesize pituitary-controlling hormones and release them into nearby blood vessels, which reach and distribute the "Turkish saddle." Harris showed that cutting of the portal vessels impeded pituitary hormone production. However, it was not until the hypothalamic hormones were discovered that Harris' theory was proved. Hypothalamic hormones, which are secreted by the hypothalamic neurons and regulate the anterior pituitary hormones (Table 1), were mostly discovered by two competiting researchers: Dr. Roger Guillemin and Dr. Andrew Schally [1–3]. Thyrotropin-releasing hormone (TRH) was the first hypothalamic hormone identified [4,5], followed by luteinizing hormone-releasing hormone ([LH-RH] or gonadotropin-releasing hormone [Gn-RH]) [6], somatostatin [7], and growth hormone-releasing hormone (GH-RH) [8,9]. The search for hypothalamic hormones by Guillemin and Schally was so competitive that it was called "the Nobel duel." Both research groups had attempted to discover corticotropin-releasing hormone (CRH), a hypothalamic hormone that is secreted by stress and releases adrenocorticotropin (ACTH) from the anterior pituitary, but without success. Although both Schally and Guillemin were awarded the Nobel Prize in 1977 [1–3], the identification of CRH needed to await the isolation of CRH from ovine brain by Vale et al. [10] and the subsequent molecular cloning of the human *CRH* gene by Shibahara et al. [11].

Another recent advance in hypothalamic research has been stimulated by the discovery of leptin, a peptide hormone secreted by adipose tissue [12]. Leptin acts on the brain, in particular on the hypothalamus, and suppresses appetite. Thus, a novel endocrine axis, the adipocyte–hypothalamic axis, has been uncovered. It has been shown that several neuropeptides in the hypothalamus, including neuropeptide Y (NPY), melanin-concentrating hormone (MCH), and α-melanocyte-stimulating hormone (α-MSH), regulate appetite and some of these neuropeptides appear to be under the control of leptin. Some types of obesity are caused by genetic abnormalities in these hormones, neuropeptides, their receptors, and processing enzymes. Obesity is closely related to the pathogenesis of hypertension, diabetes mellitus, and atherosclerosis, and is therefore one of the major challenges in medicine in the 21st century. Thus, the hypothalamus appears to have a key role in the pathogenesis of many common diseases including obesity.

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PHYSIOLOGY AND ANATOMY OF THE HYPOTHALAMUS

The hypothalamus has essential roles in the central regulation of hormone secretion in most endocrine organs, as well as a variety of autonomic functions such as the regulation of appetite, sex, temperature, water–electrolyte metabolism, circulation, emotional states, and sleep. The hypothalamus is located at the basal area of the brain between the optic chiasm (anteriorly) and the mammillary body and the posterior commissure (posteriorly) (Fig. 1). The lateral border is the internal capsule and the basis pedunculi. The dorsal limit of the hypothalamus is the horizontal level of the hypothalamic sulcus on the medial wall of the third ventricle, roughly at the horizontal level of the anterior commissure. The median eminence forms the floor of the third ventricle and constitutes the infundibulum. The hypothalamic neurons containing hypothalamic hormones release their hormones into the capillaries of the primary plexus of the hypophyseal portal system at the median eminence. The hypothalamic hormones reach the anterior pituitary lobe via the hypophyseal portal system, whereas two posterior pituitary hormones, vasopressin and oxytocin, are produced in the magnocellular neurons of paraventricular and supraoptic nuclei and are transported to the posterior pituitary lobe via the axonal transport (Fig. 2). The pituitary (hypophysis) is attached to the infundibulum.

The hypothalamus has two main roles in the endocrine regulation: (1) the production and secretion of hypothalamic hormones (Table 1), which regulate the secretion of anterior pituitary hormones from anterior pituitary lobe, and (2) the production of two posterior pituitary hormones, vasopressin and oxytocin. Hypothalamic hormones are synthesized mainly in the parvocellular neurons of the paraventricular nucleus, periventricular nucleus, and arcuate nucleus of hypothalamus. For example,

Table 1
Hypothalamic Hormones

Hypothalamic hormones	*Abbreviations*	*No. of amino acids*	*Target pituitary hormones*	*Effects*
Corticotropin-releasing hormone	CRH	41 amino acids	ACTH	(+)
Growth hormone-releasing hormone	GH-RH or GRH	40 or 44 amino acids	GH	(+)
Somatostatin (growth hormone-release inhibiting hormone)		14 or 28 amino acids	GH and TSH	(–)
Gonadotropin-releasing hormone (luteininzing hormone-releasing hormone)	Gn-RH or LH-RH	10 amino acids	LH and FSH	(+)
Thyrotropin-releasing hormone	TRH	3 amino acids	TSH and prolactin	(+)

(+), Stimulatory effects; (–), inhibitory effects.

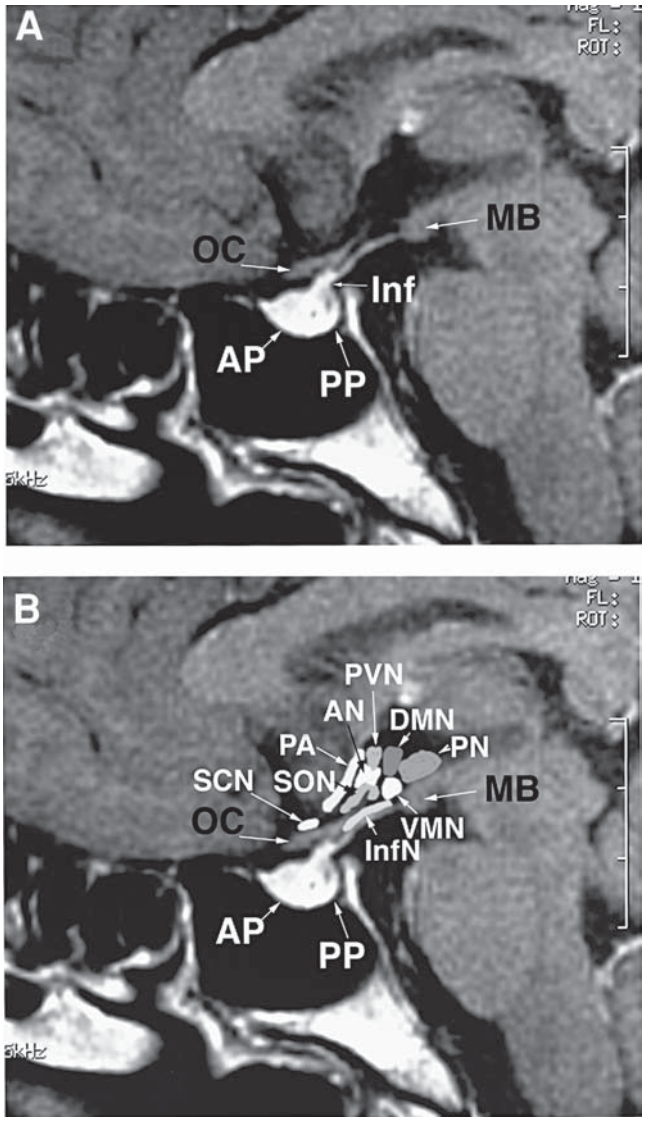

Figure 1 (**A**) Magnetic resonance imaging (MRI) of the brain of a normal subjects, and (**B**) the approximate positions of the hypothalamic nuclei. OC, Optic chiasma; AP, anterior pituitary lobe (adenohypophysis); PP, posterior pituitary lobe; Inf, infundibulum; MB, mammillary body; SCN, suprachiasmatic nucleus; AN, anterior nucleus; PA, preoptic area; PVN, paraventricular nucleus; DMN, dorsomedial nucleus; PN, posterior nucleus; VMN, ventromedial nucleus; InfN, infundibular nucleus (arcuate nucleus); SON, supraoptic nucleus. (After Carpenter MB, Sutin J. Human Neuroanatomy, 8th edit. Baltimore/London: Williams & Wilkins, 1983.)

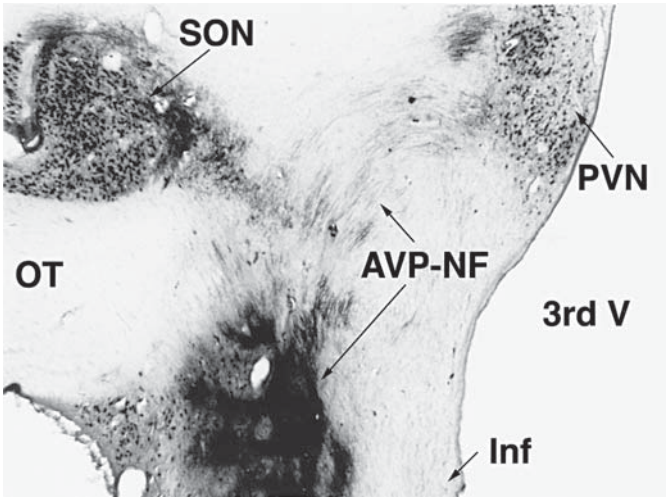

Figure 2 Immunocytochemistry of arginine vasopressin in the human hypothalamus. Vasopressin immunoreactive cell bodies are located in the paraventricular nucleus (PVN) and supraoptic nucleus (SON). Arginine vasopressin positive nerve fibers (AVP-NF) run from these nuclei toward the infundibulum (Inf) and the pituitary stalk. 3rd V, Third ventricle; OT, optic tract. (Reproduced from Takahashi K, Murakami O, Satoh F, Mouri T. The hypothalamus and neurohypophysis. In: Stefaneau L, Sasano H, Kovacs K, eds. Molecular and Cellular Endocrine Pathology. London: Arnold, 2000:45–74, with permission.)

CRH is produced in the parvocellular neurons of the paraventricular nucleus (Fig. 3). Vasopressin and oxytocin are produced separately in magnocellular neurons of the paraventricular nucleus and supraoptic nucleus of hypothalamus. Figure 2 shows vasopressin neurons in the paraventricular nucleus and supraoptic nucleus, which project nerve fibers to the infundibulum and finally to the pituitary. It is noteworthy that vasopressin is expressed also in the parvocellular cells of the paraventricular nucleus, where vasopressin is colocalized with CRH [13] (Fig. 4). Vasopressin has a vasoconstrictor action (mediated by the V1 receptor), and an antidiuretic action (mediated by the V2 receptor). In addition to these actions, vasopressin stimulates the secretion of ACTH and potentiates the CRH-stimulated ACTH release from the anterior pituitary lobe, and this action is mediated by V3 receptor (or V1b receptor) [14,15]. Vasopressin and CRH, which are colocalized together in the parvocellular neurons of the paraventricular nucleus, reach the anterior pituitary

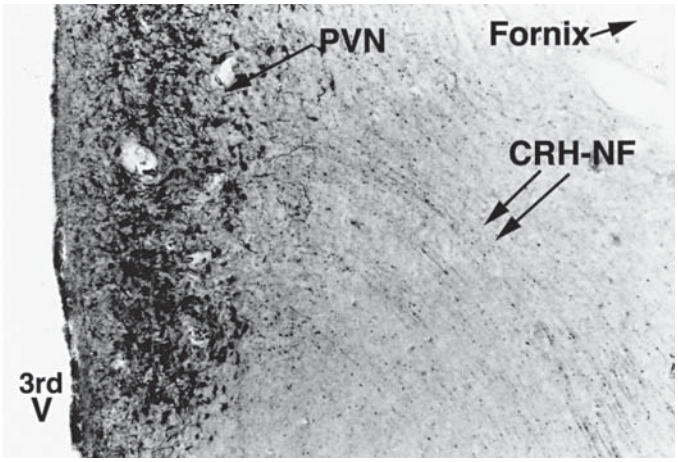

Figure 3 Immunocytochemistry of CRH in the human hypothalamus. CRH-immunoreactive cell bodies are localized in the parvocellular cells of the paraventricular nucleus (PVN). CRH-immunoreactive nerve fibers (CRH-NF) run from these cell bodies laterally and downwards. 3rd V, Third ventricle.

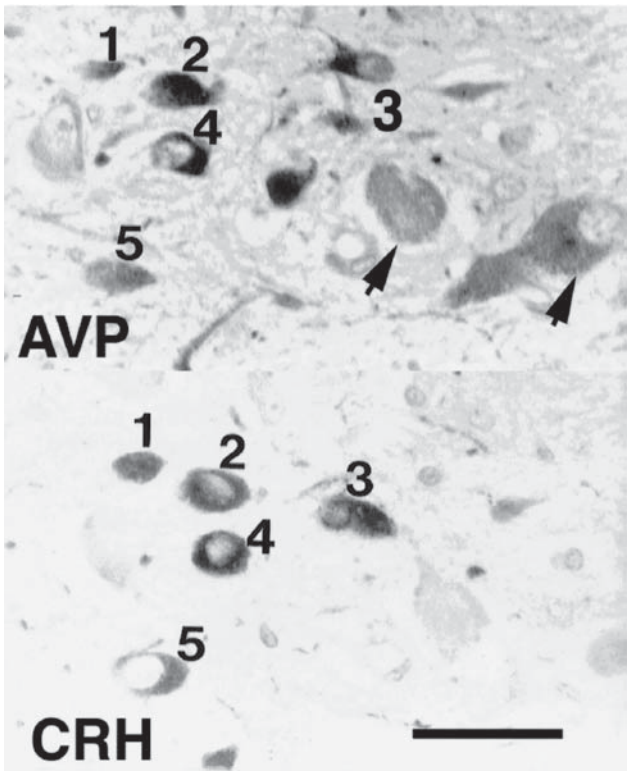

Figure 4 Colocalization of CRH and arginine vasopressin (AVP) in the parvocellular neurons of the paraventricular nucleus of human hypothalamus. CRH is localized in the parvocellular neurons of the paraventricular nucleus and colocalized with vasopressin in these neurons. Vasopressin is also localized in the magnocellular neurons of the paraventricular nucleus and supraoptic nucleus. Vasopressin in the magnocellular neurons is transported to the posterior pituitary lobe by axonal transport, whereas vasopressin in the parvocellular cells is supposed to be transported to the anterior pituitary lobe with CRH via the hypophysial portal system. The numbers indicate identical cell bodies on the serial sections. Arrows indicate AVP-positive magnocellular neurons. Bar = 50 μm.

lobe via the hypophyseal portal system and synergistically regulate the secretion of ACTH.

There are approx 20 nuclei in the hypothalamus. In addition to the paraventricular, supraoptic, and arcuate (infundibular) nuclei, the following nuclei are present: the suprachiasmatic, median preoptic, medial preoptic, lateral preoptic, anterior, diffuse supraoptic, tuberomammillary, lateral tuberal, dorsomedial, ventromedial, perifornical, posterior, medial mammillary, lateral mammillary, premammillary, and supramammillary. There is a complicated neuronal network among these nuclei, and between these nuclei and extrahypothalamic brain areas.

HYPOTHALAMIC HORMONES

Hypothalamic hormones include the following five peptide hormones: corticotropin-releasing hormone (CRH), growth hormone-releasing hormone (GRH or GH-RH), somatostatin (growth hormone-release inhibiting hormone), gonadotropin-releasing hormone (Gn-RH or luteinizing hormone-releasing hormone, LH-RH), and thyrotropin-releasing hormone (TRH) (Table 1). These hypothalamic hormones are produced in the cell bodies of neurons located in the hypothalamus and transported through nerve fibers to the median eminence (Fig. 3). Hypothalamic hormones are also produced in other brain regions and may act as neurotransmitters or neuromodulators. For example, TRH has been shown to increase locomotor activity, increase body temperature, cause shaking behavior, etc. [16].

In addition to these hypothalamic hormones, a number of bioactive substances are involved in the regulation of anterior pituitary hormone secretion [17]. These include classical neurotransmitters (e.g., noradrenaline, dopamine, acetylcholine), excitatory amino acids (e.g., glutamic acid), various neuropeptides (e.g., neuropeptide Y [NPY], calcitonin gene-related peptide [CGRP] [Fig. 5], vasoactive intestinal polypeptide [VIP], pituitary adenylate-cyclase activating polypeptide [PACAP] [Fig. 6], substance P, and endothelin-1), and bioactive gas molecules (e.g., nitric oxide [NO] and carbon monoxide [CO]). These regulatory substances (1) act on the neurons in the hypothalamus and regulate the production and/or secretion of hypothalamic hormones; (2) are released into the portal vessels, and modulate the actions of hypothalamic hormones on the anterior pituitary lobe; and (3) are produced in the anterior pituitary cells and act as paracrine or autocrine regulators in the anterior pituitary hormone secretion.

CRH CRH is a 41-amino-acid peptide originally isolated from the ovine hypothalamus [10]. Human CRH is identical to rat CRH, but the structure of human CRH differs in seven amino acids from that of ovine CRH [11]. The production and secretion of CRH in the hypothalamus are regulated by the negative feedback mechanism of ACTH from the anterior pituitary and cortisol from the adrenal cortex. Inflammatory cytokines, noradrenaline, and neuropeptides, such as NPY, also affect the production and secretion of CRH in the hypothalamus.

In human hypothalamus, CRH is localized in the parvocellular neurons of the paraventricular nucleus (Fig. 3), and colocalized with arginine vasopressin in these neurons (Fig. 4) [13]. It is known that a stimulatory effect of CRH on ACTH secretion is potentiated by arginine vasopressin. In experimental animals, adrenal insufficiency due to adrenalectomy causes increases

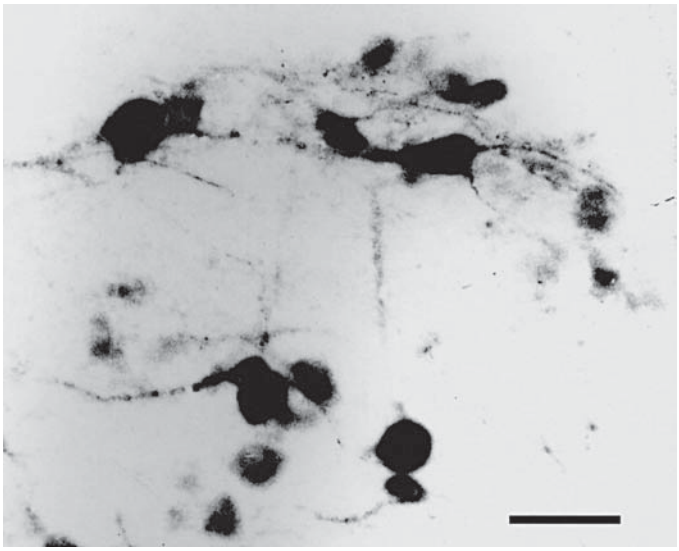

Figure 5 Immunocytochemistry of calcitonin gene-related peptide (CGRP) in the human hypothalamus (supraoptic nucleus). Bar = 50 μm. CGRP is a 37-amino-acid peptide that is produced by the alternative processing of the calcitonin gene transcript. CGRP acts as a neurotransmitter or neuromodulator, in particular, in nociception, ingestive behavior, and modulation of autonomic and endocrine systems. CGRP is expressed in the supraoptic and paraventricular nuclei of hypothalamus and anterior pituitary endocrine cells.

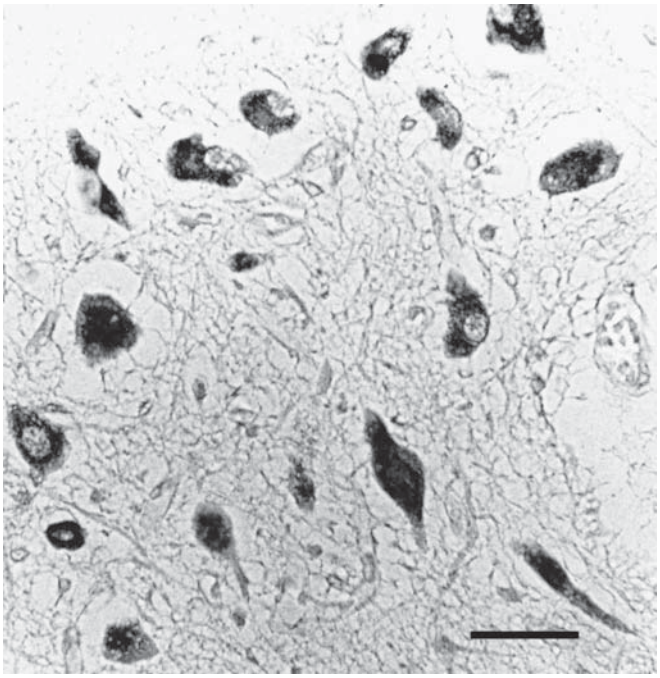

Figure 6 Immunocytochemistry of pituitary adenylate cyclase activating polypeptide (PACAP) in the human hypothalamus (paraventricular nucleus). Bar = 50 μm. PACAP is a 38- or 27-amino-acid peptide that was isolated from ovine hypothalamus on the basis of the ability to stimulate adenylate cyclase in rat pituitary cells. PACAP increases release of GH, prolactin, ACTH, and LH from superfused rat pituitary cells. PACAP is expressed in the supraoptic and paraventricular nuclei of hypothalamus.

in the expression of CRH and arginine vasopressin in these neurons, which are suppressed by glucocorticoid supplement. Patients with primary or secondary adrenal insufficiency often show a defect in water excretion accompanied by hyponatremia and elevated plasma vasopressin levels. Increased production of vasopressin in the parvocellular neurons may account for the pathogenesis of hyponatremia in these patients.

CRH is present not only in the hypothalamus but also in extrahypothalamic brain areas and peripheral tissues, such as adrenal medulla and gastrointestinal tract. CRH may act as a neurotransmitter or neuromodulator in the brain, for example, for the regulation of appetite regulation and emotion in various aspects of stress. There are at least three other members in the CRH family peptides: urocortin, stresscopin-related peptide (urocortin II), and stresscopin (urocortin III) [18–20], which are described in the subheading, Receptors for Hypothalamic Hormones and Their Mutation.

GH-RH AND SOMATOSTATIN Growth hormone (GH) in the anterior pituitary is regulated by two hypothalamic hormones: the releasing hormone is growth hormone-releasing hormone (GH-RH or GRH) and the release-inhibiting hormone is somatostatin.

GH-RH was isolated from the tumor tissue of a pancreatic islet cell tumor in a patient with acromegaly [8,9]. Two major forms are found: a 44-amino-acid peptide and a shorter 40-amino-acid peptide identical to the N-terminal 40 amino acids of GH-RH-(1–44), both of which are also found in the hypothalamus. Somatostatin is a 14-amino-acid peptide that was isolated from sheep hypothalami in the search for the hormone responsible for stimulating the release of GH from the anterior pituitary [7]. Somatostatin inhibits the secretion of both GH and TSH from the anterior pituitary lobe. A larger molecular form of somatostatin consisting of 28 amino acids (somatostatin-28), a precursor of somatostatin-14, is also present. Somatostatin-14 corresponds to a C-terminal 14-amino-acid portion of somatostatin-28.

GH-RH immunoreactive cell bodies and somatostatin-immunoreactive cell bodies are found in the infundibular nucleus of the human hypothalamus [21,22], although these two peptides are not colocalized in the same neurons. GH-RH containing cell bodies are localized in an area more dorsal than that of somatostatin-containing cell bodies. GH-RH and somatostatin are also expressed in extrahypothalamic brain areas and peripheral tissues, such as gastrointestinal tract, pancreatic islets, and adrenal medulla.

In addition to GH-RH and somatostatin, a number of factors such as free fatty acids, acetylcholine, amino acids, opiates, glucocorticoids, and some neuropeptides also have direct or indirect effects on GH release. GH secretagogues were discovered as a series of small peptide derivatives of Met-enkephalin, which selectively stimulated GH secretion from pituitary cells [23]. GH secretagogues act via a specific G-protein-coupled receptor: the GH secretagogue receptor [24], not via the GH-RH receptor. Ghrelin is a recently discovered endogenous ligand for GH

secretagogue receptor [25]. Ghrelin is a 28-amino-acid peptide with an octanoyl group on the third N-terminal amino acid serine, and is expressed in the arcuate nucleus of hypothalamus and the endocrine cells of stomach. Appetite-stimulating actions of ghrelin are described in the subheading, Appetite Regulation, Obesity, and Anorexia Nervosa.

Gn-RH (LH-RH) LH-RH is a 10-amino-acid peptide that stimulates secretion of two gonadotropins: LH and follicle-stimulating hormone (FSH), from the anterior pituitary [6]. Therefore LH-RH is also called gonadotropin-releasing hormone (Gn-RH). The Gn-RH precursor generates another peptide called Gn-RH-associated peptide (GAP). GAP stimulates gonadotropin release from rat anterior pituitary cells in culture [26].

Gn-RH-expressing neurons migrate during development from their birthplace on the medial side of the olfactory placode into the brain [27]. In the adult hypothalamus, Gn-RH-positive cell bodies are most numerous in the ventral and basal hypothalamus [28]. Gn-RH-positive cell bodies extend ventrally as far as the median eminence and infundibular stalk and caudally as far as the mammillary complex. Gn-RH-positive nerve fibers were observed in the median eminence and the organum vasculosum of the lamina terminalis. A large proportion of Gn-RH fibers continue uninterrupted through the internal zone of the median eminence and infundibular stalk to enter the posterior pituitary. The physiological roles of Gn-RH transported to the posterior pituitary remain to be determined. In addition, there are extrahypothalamic projections of Gn-RH fibers to the habenular complex, the amygdaloid complex, hippocampus, midbrain, and cingulate cortex that may be related to the complex mechanism of reproduction.

Gn-RH is secreted in a pulsatile fashion, which results in the pulsatile secretion of gonadotropins from the anterior pituitary. An increase in pulsatile release of Gn-RH is essential for the onset of puberty [29]. Gn-RH secretion and Gn-RH mRNA expression are influenced by estrogen, progesterone, and some neurotransmitters, such as γ-aminobutyric acid (GABA), NPY, opioids, glutamate, and noradrenaline [27,29]. Estrogen and progesterone increase Gn-RH mRNA in the hypothalamus of rats. GABA reduces Gn-RH mRNA expression levels and its release.

TRH TRH was the first discovered hypothalamic hormone that consists of three amino acids (pGlu-His-Pro-NH_2) [4,5]. TRH is the shortest peptide among hypothalamic hormones. TRH stimulates the secretion of both TSH and prolactin from the anterior pituitary lobe. TRH-containing neurons are found in the suprachiasmatic–preoptic nucleus, parvocellular subdivision of the paraventricular nucleus, perifornical area, dorsomedial nucleus, and lateral hypothalamus. TRH is present not only in hypothalamus but also in other regions of brain, and may act as a neurotransmitter or neuromodulator. TRH increases locomoter activity, increases body temperature, causes shaking behavior, decreases food intake, among other effects [16].

HYPOTHALAMIC CONTROL OF PROLACTIN SECRETION

Prolactin release from the pituitary lactotropes is regulated by a tonic inhibitory control of dopamine derived from the hypothalamus [30]. The lesions in hypothalamus or pituitary stalk result in the decreased delivery of hypothalamic hormones to the anterior pituitary gland, and cause hypofunction of anterior pituitary hormones, except for prolactin. Prolactin secretion is rather increased in the lesions of hypothalamus or pituitary stalk because of decreased delivery of dopamine to the anterior pituitary.

Several candidates for prolactin-releasing factor (PRF) have been proposed; these include TRH, vasoactive intestinal polypeptide (VIP) [31], peptide histidine methionine (PHM), galanin, oxytocin, and prolactin-releasing peptide (PrRP) [32]. Although all these peptides have a prolactin-releasing activity, a physiological PRF has not been identified. VIP is a potent vasodilator peptide consisting of 28 amino acids, which are abundant in the gastrointestinal tract and the brain. The VIP precursor generates another bioactive peptide called PHM (peptide histidine isoleucine [PHI] in pig and rat) by posttranslational proteolytic processing [33]. PHM has a structurally similarity to VIP and similar biological actions such as vasodilator action and prolactin-releasing activity.

RECEPTORS FOR HYPOTHALAMIC HORMONES AND THEIR MUTATION

Receptors for hypothalamic hormones —CRH, GH-RH, somatostatin, TRH, and Gn-RH—belong to the GTP-binding protein (G-protein)-coupled receptor superfamily. They have a common structure with seven transmembrane domains. Receptors for each peptide consist of several subtypes, which have different pharmacological properties. For example, there are at least five somatostatin receptor subtypes: SSTR1, SSTR2, SSTR3, SSTR4, and SSTR5. SSTR5 is a receptor subtype that is predominantly expressed in the pituitary.

CRH receptors consist of two subtypes: CRH receptor type 1 and 2. CRH receptors type 1 are further divided into CRH receptor type 1a, 1b, 1c, 1d, 1e, 1f, 1g, and 1h. CRH receptors type 2 are also divided into CRH receptor type 2α, 2β, and 2γ. Both CRH receptor type 1 and type 2 are expressed in various organs including the brain. In particular, CRH receptor type 1 is expressed in the anterior pituitary lobe and mediates the CRH effect on ACTH release. CRH receptors type 2 are widely expressed in various organs including brain and heart, and may be important in the regulation of the recovery phase of the stress response. CRH receptors type 2 mediates the countershock responses, such as hypotensive, cardioprotective, anxiolytic, and anorexic responses. CRH and urocortin, a CRH family peptide consisting of 40 amino acids, act on both CRH receptors type 1 and 2. On the other hand, two newly identified CRH family peptides, stresscopin (urocortin III) and stresscopin-related peptide (urocortin II), are specific ligands for CRH receptor type 2 [18–20].

Some mutations in the genes coding these receptors for hypothalamic hormones result in hypopituitary function. There have been several case reports on hypogonadotropic hypogonadism [34,35], short statue (dwarfism) [36–38], or central hypothyroidism [39], which were caused by the mutations in the Gn-RH receptor, the GH-RH receptor, or the TRH receptor (Table 2).

The signals from the hypothalamic hormone receptors are transmitted by the G proteins to the second messengers. For example, GH-RH uses cAMP as a second messenger to stimulate GH secretion and proliferation of normal pituitary somatotrophs. The signal of GH-RH is transmitted from the GH-RH receptors to the G protein. The G proteins involved in the signal transduction are heterodimers consisting of α, β, and γ subunits. Activity of adenylyl cyclase is regulated by at least two G pro-

Table 2
Mutations in the Receptors for Hypothalamic Hormones

Hypothalamic hormones	*Symptoms and signs*	*Refs.*
Gn-RH	hypognadotropic hypogonadism	34,35
GH-RH	short statue (dwarfism)	36–38
TRH	central hypothyroidism	39

teins; Gs is responsible for the stimulation of catalytic activity, whereas Gi mediates the inhibition of this enzyme. Mutations that lead to constitutive activation of $G_{s\alpha}$ have been identified in a subset of human GH-secreting pituitary tumors [40,41].

McCune–Albright syndrome is a sporadic disease characterized by cutaneous hyperpigmentation, polyostotic fibrous dysplasia, and multiple endocrinopathies, including precocious puberty, hyperthyroidism, hypercortisolism, GH-secreting pituitary adenoma, and hyperprolactinemia. McCune–Albright syndrome is caused by the mutations in the gene encoding $G_{s\alpha}$ protein, which lead to constitutive activation of $G_{s\alpha}$ and increased cAMP formation [42,43]. These diverse metabolic abnormalities actually share the involvement of cells that respond to extracellular signals through activation of the hormone-sensitive adenylyl cyclase system. Only GH-secreting pituitary tumors but not other types of pituitary tumors have been described in patients with McCune–Albright syndrome, although somatic mutations of Gs may occur in all pituitary cell lines. This may be caused because only the somatotrophs respond with uncontrolled proliferation [42].

HYPOTHALAMIC HORMONE–SECRETING TUMORS

Hypothalamic hormone–secreting tumors arise mostly from the peripheral tissues. There are a limited numbers of case reports on hypothalamic hormone–secreting tumors in the hypothalamus or in the area near the hypothalamus. For example, hypothalamic hamartoma secreting Gn-RH is one of the causes of precocious puberty [44]. There are cases reports on intrasellar or hypothalamic gangliocytoma secreting CRH or GH-RH [45, 46]. The majority of hypothalamic hormone–secreting tumors arise from the peripheral tissues belonging to the APUD system, such as the lung (carcinoid tumor), thymus (thymoma), pancreatic islet, adrenal medulla (pheochromocytoma), and sympathetic ganglia (neuroblastoma and ganglioneuroblastoma). But many ectopic hormones are also secreted by apparently non-APUD cells [47].

Gn-RH–Secreting Hypothalamic Hamartoma Hamartoma of the central nervous system is a congenital malformation characterized as heterotropic and hyperplastic tissue that is usually encountered at the base of the brain, the interpeduncular cistern, or within the hypothalamus, and located in proximity to the tuber cinereum and the mammillary bodies [48]. Hypothalamic hamartomas are often associated with precocious puberty. Thirty-seven (74%) of the 50 tissue-proved cases of hamartomas found in the literature showed precocious puberty [48]. Mechanisms underlying this association remain to be determined. One possibility is that the pressure applied by the tumor directly to the hypothalamic centers caused abnormal function. Neuronal stimulation via myelinated fibers connecting the hamartoma to the hypothalamus has also been proposed. Another possibility is that neurons in the aberrant tissue secrete a hormone or hormones that prematurely activate the pituitary–gonadal axis. There are reports on the presence of Gn-RH in the hypothalamic hamartomas obtained from patients with precocious puberty [49,50].

GH-RH–Secreting Tumors GH-RH–producing tumors with acromegaly arise both from intracranial tissues and extracranial tissues. The association of a gangliocytoma in the hypothalamus with acromegaly has been reported since the 1950s. Intrasellar or hypothalamic gangliocytomas with acromegaly have been shown to secrete GH-RH [46].

As GH-RH was originally isolated from the tumor tissue of a pancreatic islet cell tumor in a patient with acromegaly [7,8], GH-RH is produced by various tumors, including islet cell pancreatic tumors, small cell lung carcinomas, bronchial adenocarcinomas, carcinoids, pancreatic adenocarcinomas, breast carcinomas, ovarian carcinomas, pheochromocytomas, medullary thyroid carcinomas, and ganglioneuroblastomas [51]. Although the tumor tissues contain GH-RH, most of the patients with these tumors are free of acromegaly, a symptom that is due to the GH hypersecretion. In only a small number of patients with these tumors, acromegaly occurred clinically [52], possibly because the hypersecretion of GH-RH from the tumor is large enough to cause hypersecretion of GH from the pituitary in only a limited number of cases. The most frequent GH-RH–secreting extracranial tumors with acromegaly are carcinoids (69% of the cases), most often located in the lung (78%) or gastrointestinal tract (11%), followed by islet cell tumors (34%). One cystic bronchial adenoma, one pheochromocytoma, and one paraganglioma with acromegaly were also reported [52].

CRH-Secreting Tumors There is one case report of Cushing's disease associated with an intrasellar gangliocytoma producing CRH [45]. Most CRH-secreting tumors, however, arise from the peripheral tissues. Ectopic CRH secretion is usually accompanied by ectopic ACTH secretion. In other words, CRH expression is detectable in many of ectopic ACTH-secreting tumors, such as bronchial carcinoids, neuroendocrine tumors of thymus, small cell carcinomas of the lung, colon carcinomas, nephroblastomas, and thyroid medullary carcinomas. In these ectopic ACTH/CRH-secreting tumors, plasma ACTH levels are elevated, whereas plasma CRH levels are rarely elevated, suggesting that the tumor CRH is not likely to act on the pituitary, but may affect the ACTH secretion from the tumor as an autocrine/paracrine regulator. Two cases of pure CRH-containing tumors have been reported: metastatic carcinoma of the prostate [53] and thyroid medullary carcinoma [54].

It is well known that pheochromocytomas express a variety of neuropeptides and vasoactive peptides, such as NPY, VIP, GH-RH, somatostatin, CGRP, and so forth. Pheochromocytomas also express ACTH and CRH [55]; ACTH and CRH are detectable in tumor tissues of most pheochromocytomas by radioimmunoassay. Plasma levels of ACTH and CRH are, however, not elevated in most cases of pheochromocytomas, which are therefore free from Cushing's syndrome. A very limited number of patients with pheochromocytomas show the hormone excess syndromes, such as Cushing's syndrome [56].

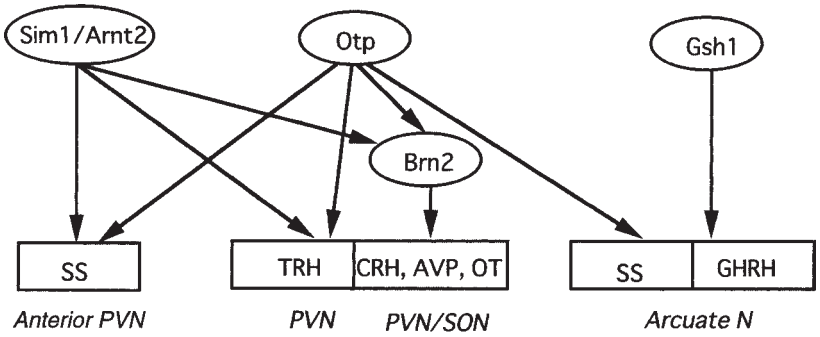

Figure 7 Relationship between transcriptional factors and hypothalamic hormones in the development of hypothalamus. SS, Somatostatin; TRH, thyrotropin-releasing hormone; CRH, corticotropin-releasing hormone; AVP, arginine vasopressin; OT, oxytocin; GHRH, growth hormone-releasing hormone; PVN, paraventricular nucleus; SON, supraoptic nucleus.

Somatostatin-Secreting Tumors (Somatostatinomas) Main symptoms and signs of somastostatinoma are insulin-sensitive, nonketosis-prone diabetes, steatorrhea, and cholelithiasis. About 47% of somatostatinomas arise in the pancreatic islets [57]. Somatostatin overproduction was also found in the carcinoid tumors arising in the small intestine, medullary thyroid carcinomas, pheochromocytomas, small cell carcinomas of the lung, and retinoblastomas. The incidence of somatostatinoma syndrome is, however, not so high (18.5% in pancreatic somatostatinomas and 2.5% in extrapancreatic somatostatinomas). About half cases of somatostatinomas are malignant.

DEVELOPMENT OF HYPOTHALAMUS AND TRANSCRIPTIONAL FACTORS

Recent studies in gene knockout mice have revealed that certain transcriptional factors are essential for the development of the hypothalamic magnocellular and parvocellular neurosecretory system. These transcriptional factors include Brain-2 (*Brn-2*), *Sim1*, Orthopedia (*Otp*), *Arnt2*, and *Gsh-1*.

Brn-2 belongs to the class III *POU* gene family [58,59]. The POU domain is a conserved DNA-binding motif, which is about 150-amino-acid residues long and consists of two highly conserved regions separated by a 15–20-amino-acid residue linker region. *Brn-2* is expressed in specific regions of the mouse brain including the paraventricular nuclei of the hypothalamus, and binds to and activates the CRH promoter. In homozygous *Brn-2* mutant embryos, migratory precursor cells for neurons of the paraventricular nuclei and the supraoptic nuclei of the hypothalamus, die at E 12.5. All homozygous mutants suffered mortality within 10 d after birth, possibly because of the defect in the secretion of CRH and vasopressin. In heterozygous mice, which had no developmental or histological abnormalities, the levels of expression of vasopressin and oxytocin in the hypothalamus were half those of wild-type mice. Thus, *Brn-2* is essential for the development of the magnocellular and parvocellular neurons of the paraventricular nucleus and supraoptic nucleus, which secrete CRH, vasopressin, and oxytocin.

Sim1 and *Arnt2* are members of basic helix–loop–helix (*bHLH*) *–PAS* (a conserved sequence among *Per*, *AhR/Arnt*, and *Sim*) family of transcription factors [60,61]. These transcription factors are mainly classified into two groups: the *AhR* (arylhydrocarbon receptor) group and *Arnt* (*AhR* nuclear translocator) group. The *AhR* group, which includes *Sim1*, does not dimerize with themselves or other members within the group, but they do heterodimerize with members of the *Arnt* group. *Sim1* is expressed in the paraventricular, anterior periventricular, and supraoptic nuclei during the development of the hypothalamic–pituitary axis. The expression of *Arnt2* is limited to the neural tissue, whereas *Arnt* is broadly expressed in various tissues. $Sim1^{-/-}$ mice and $Arnt2^{-/-}$ mice shows similar phenotypes. They die shortly after birth. The supraoptic nuclei and paraventricular nuclei are hypocellular in both types of mice. At least five distinct types of secretory neurons, which secrete oxytocin, vasopressin, TRH, CRH, and somatostatin, respectively, are absent in the paraventricular, anterior periventricular, and supraoptic nuclei of $Sim1^{-/-}$ mice. Similarly in the mutant *Arnt2* mice, secretory neurons of oxytocin, vasopressin, CRH, and somatostatin are completely absent in the supraoptic and paraventricular nuclei. During development of the *Sim1* mutant or *Arnt2* mutant hypothalamus, the prospective paraventricular/supraoptic region fails to express *Brn-2*, suggesting that *Sim1* and *Arnt2* function upstream to maintain *Brn-2* expression.

Otp is a highly conserved homeodomain-containing factor that is expressed during embryonic development in neurons giving rise to the paraventricular, supraoptic, anterior periventricular, and arcuate nuclei of hypothalamus [62]. In homozygous $Otp^{-/-}$ mice, paraventricular, supraoptic, and anterior periventricular nuclei were absent, whereas arcuate nucleus was impaired but present. $Otp^{-/-}$ mice fail to express CRH, TRH, vasopressin, oxytoxcin, and somatostatin, and die soon after birth. They retained a normal expression of GH-RH in the arcuate nucleus.

Gsh-1 is a homeobox gene that is essential for GH-RH gene expression in the arcuate nucleus [63]. $Gsh\text{-}1^{-/-}$ mice exhibit extreme dwarfism, sexual infantilism, and significant perinatal mortality, with small-sized and hypocellular pituitary.

A summary of the relationship between these transcriptional factors and hypothalamic hormones is shown in Fig. 7. On the other hand, there have been no reports on human cases of abnormal hypothalamo–pituitary functions due to mutations of these transcriptional factors, although there is a possibility that such human cases may be demonstrated in the future.

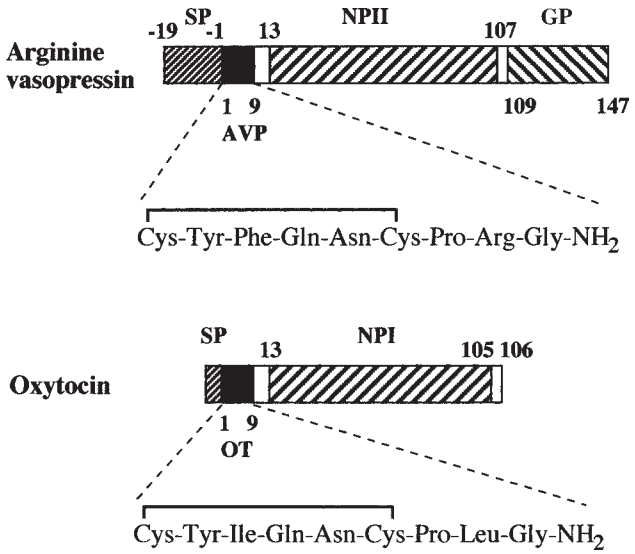

Figure 8 Schematic representation of the precursors of arginine vasopressin (AVP) and oxytocin (OT), and the amino acid sequences of AVP and OT. SP, Signal peptide; NPI, neurophysin I; NPII, neurophysin II; GP, glycoprotein.

NEUROHYPOPHYSIS

The human neurohypophysis is described as consisting of three parts: (1) the median eminence of the hypothalamic tuber cinereum; (2) the infundibular stem, which, together with the portion of adenohypophysis that surrounds it (part tuberalis), constitutes the pituitary or hypophysial stalk; and (3) the pars nervosa (posterior neural lobe) or infundibular process [64].

Vasopressin and oxytocin are produced in the magnocellular neurons of the paraventricular and supraoptic nuclei, transported by the axonal transport to the posterior pituitary lobe (posterior neural lobe), and released there into the circulation (Fig. 2). The posterior pituitary lobe consists mainly of nerve fibers of the hypothalamic neurons, pituicytes, which are thought to be supportive cells (a kind of glial cells), and the vascular tissues. Anterior pituitary cells, mostly ACTH cells, invade into and are scattered in the posterior pituitary lobe.

Vasopressin is also called antidiuretic hormone (ADH) following its main actions on the kidney. In human, rat, and mouse, vasopressin has an arginine residue at position 8, and is therefore called arginine vasopressin, while lysine vasopressin found in pig has a lysine residue at position 8. The schematic structures of precursors of vasopressin (arginine vasopressin) and oxytocin are shown in Fig. 8. The cDNAs encoding the precursors of oxytocin and vasopressin code other proteins, neurophysin I and neurophysin II, respectively. Neurophysins are generated by proteolytic processing of the precursors in secretory granules, and are supposed to have important roles in the axonal transport of oxytocin and vasopressin from the hypothalamus to the neurohypophysis.

The most important factors that regulate the production and the secretion of vasopressin are plasma osmolarity and circulating blood volume. Increases in plasma osmolarity augment plasma levels of vasopressin. Decreases in circulating blood volume, such as hemorrhagic shock, release vasopressin from the neurohypophysis and elevate plasma levels. Several neurotransmitters and neuropeptides, such as angiotensin II, natriuretic peptides, and endorphin, regulate the production and secretion of vasopressin in the hypothalamus and the neurohypophysis. Acetylcholine stimulates vasopressin secretion, whereas β-adrenergic agonists inhibit the vasopressin secretion. Angiotensin II and endorphin release vasopressin, while natriuretic peptides inhibit its secretion. Vasopressin actions are mediated by tissue-specific G-protein-coupled receptors, which are currently classified into V1 vascular (vasoconstrictor action), V2 renal (antidiuretic action), and V3 pituitary (or V1b) (stimulation of ACTH release) subtypes [14,15,65], as described in the previous subheading, Physiology and Anatomy of the Hypothalamus.

The secretion of oxytocin is stimulated by the so-called "milk let-down reflex." The stimulus of suckling causes a neurogenic reflex that is transmitted from afferent nerve endings in the nipple to the hypothalamus, where the secretion of oxytocin is stimulated. Oxytocin causes contraction of the myoepithelial cells in the breast and stimulates the secretion of milk. Oxytocin also has a uterus-contracting action. These actions are mediated by the oxytocin-specific G-protein-coupled receptor. The physiological significance of oxytocin in men and nonpregnant women remains to be determined. Recent studies, however, have shown that oxytocin is involved in endocrine and neuroendocrine regulation through receptor-mediated actions exerted on the heart, vasculature, and kidneys [66–69]. For example, oxytocin receptor is expressed in the heart, and oxytocin has negative and chronotropic effects on cardiac atrium. In contrast to vasopressin, diseases in which oxytocin plays a major role are rarely known.

OVERVIEW OF DISEASES OF THE HYPOTHALAMUS AND NEUROHYPOPHYSIS

Diseases of the hypothalamus comprise tumors, inflammatory and infectious diseases, and genetic disorders. Tumors found in the hypothalamus include craniopharyngioma, germinoma, teratoma, meningioma, glioma (Fig. 9), and so forth. Some rare tumors that secrete hypothalamic hormones and much rarer cases of hypothalamic hormone receptor mutations are described in the previous sections. Inflammatory diseases include sarcoidosis, histiocytosis, infundibuloneurohypophysitis, and so forth. Infectious diseases such as meningitis (tuberculous, bacterial, viral, or fungal) were also found in the hypothalamus and neurohypophysis.

Hypothalamic diseases cause a variety of symptoms and signs, depending on the site of the lesion. These include sexual abnormalities (hypogonadism or precocious puberty), abnormalities in electrolyte–water metabolism (diabetes insipidus, hypernatremia, or hyponatremia), hypofunction of the anterior pituitary, psychic disturbance, abnormalities in appetite (hyperphagia and obesity or anorexia), emaciation, thermodysregulation, sleep disorders (e.g., narcolepsy), and sphincter disturbance. Diseases related to diabetes insipidus, hypogonadism, obesity, and abnormal appetite, and the sleep disorder (narcolepsy), are described in later sections. Destructive lesions of the pituitary stalk cause diabetes insipidus and hypofunction of anterior pitui-

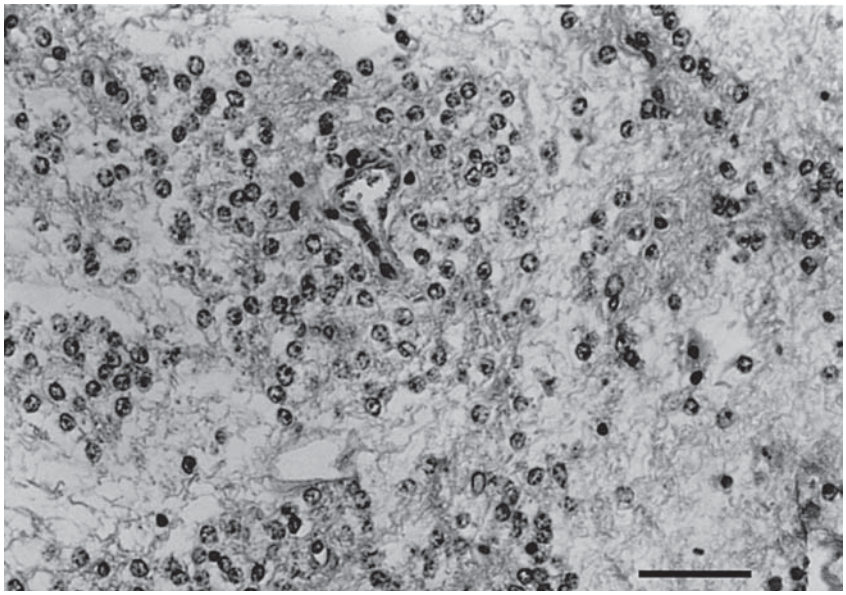

Figure 9 Astrocytoma in the hypothalamus (H&E). Bar = 50 μm. (Courtesy of Dr. H. Ikeda, Department of Neurosurgery, Tohoku University School of Medicine, Sendai, Japan.)

tary except for prolactin. These include rupture after head injury, surgical transection, tumor, and granuloma. Diabetes insipidus develops, depending on the level at which the stalk has been sectioned. If the stalk is cut at the level close to the hypothalamus, diabetes insipidus almost always occurs. If it is cut at the lower level, the incidence is less.

DISEASES DUE TO DYSFUNCTION IN VASOPRESSIN SECRETION

DIABETES INSIPIDUS Diabetes insipidus is a disease characterized by polydipsia and polyuria. Diabetes insipidus is caused by deficient production of vasopressin in the hypothalamus (neurogenic or central diabetes insipidus) or the deficient action of vasopressin in the renal tubular cells (nephrogenic diabetes insipidus).

Central Diabetes Insipidus Central diabetes insipidus is classified into three categories: familial, secondary, and idiopathic, according to the causes (Table 3). Familial diabetes insipidus is a very rare disorder and is characterized by autosomal dominant inheritance. Secondary diabetes insipidus is caused by tumors, inflammatory diseases, infection, and trauma that damage the hypothalamic–neurohypophysial system. Tumors and inflammatory diseases in the hypothalamus and neurohypophysis are described in the following sections.

Autosomal Dominant Familial Diabetes Insipidus Missense mutations of the vasopressin–neurophysin II gene have been identified in some families with familial diabetes insipidus. A single base substitution was reported in one of two alleles of the vasopressin–neurophysin II gene in families with familial diabetes insipidus [70,71]. These mutations result in one amino acid substitution in the neurophysin II moiety (Ser to Gly at amino acid position 57 in the neurophysin II moiety [70]; and Val to Gly at amino acid position 17 in the neurophysin II moiety [71]). Neurophysins bind their associated peptide hormones, vasopressin and oxytocin, after proteolytic processing of the precursor. The amino acid substitution in neurophysin II may result in its conformational change. Such changes may impair functions of neurophysin II—the protecting action for arginine vasopressin from proteolytic degradation and the assisting action of arginine vasopressin in its axonal transport. Moreover, the mutated neurophysin II may impair the function of normal neurophysin II molecules, possibly by a heterodimer formation.

A mutation was also found in the gene region encoding the vasopressin signal peptide. A point mutation causes a substitution of threonine for alanine at the last amino acid of the signal

Table 3
Classification of Neurogenic Diabetes Insipidus

Familial diabetes insipidus
Autosomal dominant
Wolfram syndrome
Secondary diabetes insipidus
Tumors
Primary tumors (craniopharyngioma, suprasellar germinoma, glioma, etc.)
Metastatic carcinomas (lung, breast, leukemia, etc.)
Anterior pituitary tumors (mostly postoperative)
Granulomatous diseases (sarcoidosis, Langerhans' cell histiocytosis, etc.)
Lymphocytic hypophysitis
Lymphocytic infundibuloneurohypophysitis
Infection
Trauma
Idiopathic diabetes insipidus (lymphocytic infundibuloneurohypophysitis?)

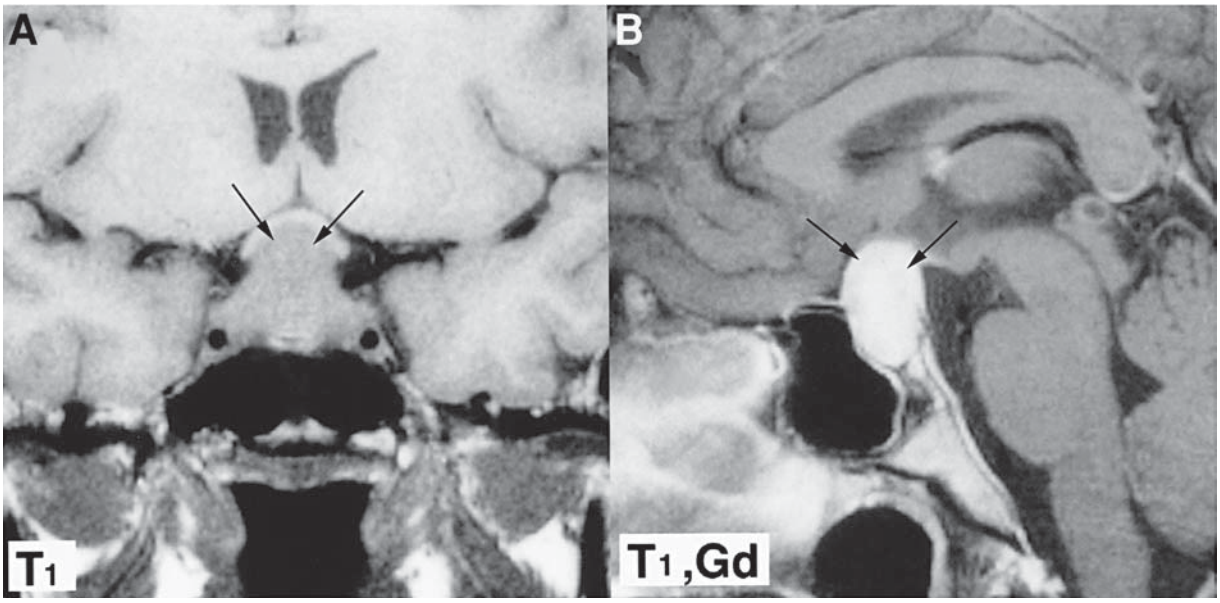

Figure 10 MRI in a patients with lymphocytic hypophysitis. Arrows indicates pituitary gland with lymphocytic hypophysitis. (**A**) Coronal T1-weighted MRI scan. (**B**) Sagittal T1-weighted MRI scan after gadolinium enhancement. (Reproduced from Takahashi K, Murakami O, Satoh F, Mouri T. The hypothalamus and neurohypophysis. In: Stefaneau L, Sasano H, Kovacs K, eds. Molecular and Cellular Endocrine Pathology. London: Arnold, 2000:45–74, with permission from Arnold.)

peptide in these patients [72–74]. The signal peptide directs the precursor protein to enter the endoplasmic reticulum, where the proteolytic cleavage of the precursor occurs. The amino acid change possibly alters the cleavage of the signal peptide and results in inefficient processing. Thus, autosomal dominant central diabetes insipidus is caused by several mechanisms.

The mutant arginine vasopressin–neurophysin II complex may accumulate slowly in the magnocellular neurons and lead to the death of these neurons [75]. Autopsy studies of patients with autosomal dominant diabetes insipidus shows a markedly subnormal number of magnocellular neurons and associated moderate gliosis [76,77].

Wolfram Syndrome Wolfram syndrome is an autosomal recessive neurodegenerative disorder associated with juvenile-onset nonimmune insulin-dependent diabetes mellitus, progressive optic atrophy, sensorineural deafness, and diabetes insipidus, and also known as DIDMOAD (Diabetes Insipidus, Diabetes Mellitus, Optic Atrophy, and Deafness) [78]. Patients present with diabetes mellitus followed by optic atrophy in the first decade, central diabetes insipidus and sensorineural deafness in the second decade, dilated renal outflow tracts early in the third decade, and multiple neurological abnormalities early in the fourth decade [78]. Central diabetes insipidus occurred in about 73% of the patients [79]. A wolfram gene (*WFS1*) has been mapped to chromosome 4p16.1 [80]. The *WFS1* encodes an 890-amino-acid protein, which is a membrane glycoprotein and localizes in the endoplasmic reticulum [81]. *WFS1* is widely expressed in the brain, including hippocampus, amygdaloid body, and hypothalamus, consistent with a variety of neurological manifestations and central diabetes insipidus.

Idiopathic Diabetes Insipidus, Lymphocytic Infundibuloneurohypophysitis, and Lymphocytic Hypophysitis Idiopathic diabetes insipidus comprises approx 30% of central diabetes insipidus. Lymphocytic infundibuloneurohypophysitis has been proposed as an important cause of what was previously considered to be idiopathic diabetes insipidus by Imura et al. [82]. On the other hand, lymphocytic hypophysitis is a rare inflammatory disease that primarily affects adenohypophysis and is probably caused by autoimmunity (Figs. 10 and 11). Approximately 19% of the patients with lymphocytic hypophysitis, however, have diabetes insipidus [83]. Lymphocytic infundibuloneurohypophysitis and lymphocytic hypophysitis are described in details in the later subheading, Inflammation in the Hypothalamus and Neurohypophysis.

Nephrogenic Diabetes Insipidus Congenital nephrogenic diabetes insipidus is a hereditary disorder characterized by the inability of the kidney to concentrate urine in response to arginine vasopressin. This disease is caused by mutations in the V2 receptor gene (X-linked recessive trait) or the gene of the renal water channel, aquaporin-2 (*AQP2*) (autosomal recessive trait).

The renal V2 receptor mediates anti-diuresis by activation of adenylate cyclase in the distal parts of the nephron. In most cases, congenital nephrogenic diabetes insipidus was inherited in an X-linked recessive mode, and caused by mutations in the V2 receptor gene, which is localized to the long arm of the X chromosome. Rosenthal et al. reported a case of congenital nephrogenic diabetes insipidus who has a deletion in the open reading frame of the V2 receptor gene, causing a frame shift and premature termination of translation in the third intracellular loop of the receptor protein [84].

The activation of the V2 receptor on renal collecting tubules stimulates adenylate cyclase via Gs and promotes the cAMP-mediated incorporation of AQP into the luminal surface of these cells. There are at least ten members of AQP: AQP0-AQP9. AQP2 is the vasopressin sensitive water channel in renal collecting ducts. Nephrogenic diabetes insipidus with autosomal reces-

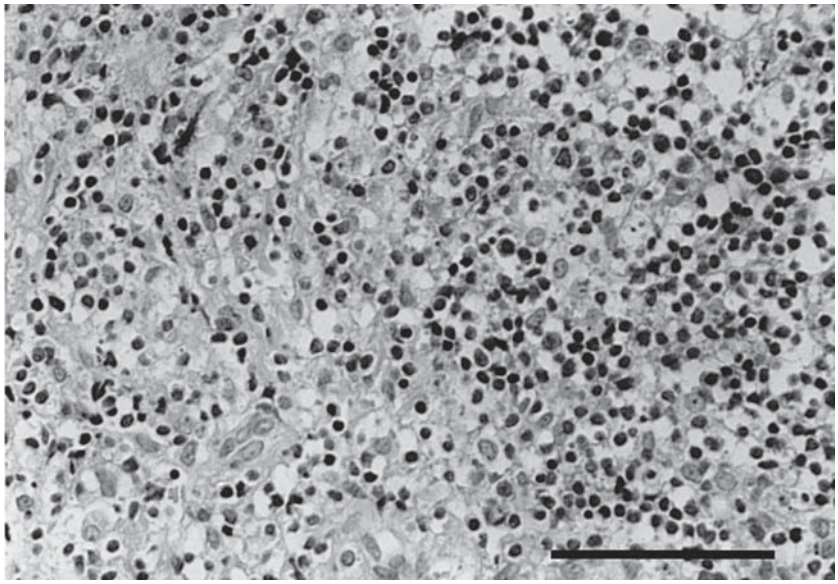

Figure 11 Lymphocytic hypophysitis (H&E). Bar = 50 μm. (Courtesy of Dr. H. Ikeda, Department of Neurosurgery, Tohoku University School of Medicine, Sendai, Japan.)

sive inheritance is caused by compound heterozygote or homozygosity for mutations in the *AQP2* gene [85,86].

SYNDROME OF INAPPROPRIATE ADH SECRETION (SIADH) SIADH is a disorder characterized by hyponatremia and impaired water excretion in the absence of hypovolemia, hypotension, or a deficiency of cardiac, renal, thyroid, or adrenal function. This disorder is caused by continual release of arginine vasopressin in spite of a subnormal plasma osmolarity. The diseases most often associated with SIADH are shown in Table 4. The most common causes for SIADH are malignant diseases, especially small cell or oat cell carcinomas of the lung, which produce and secrete arginine vasopression. The other malignant tumors associated with SIADH include carcinomas of the pancreas, duodenum, bladder and prostate, thymomas, lymphomas, and Ewing's sarcomas. Nonmalignant pulmonary diseases are associated with SIADH, probably because hypoxia, hypercapnea, or increased intrathoracic pressure may stimulate the release of vasopressin. The lesions in the brain as shown in the Table 4 may directly or indirectly stimulate the hypothalamo–neurohypophysis to secrete vasopressin. Several drugs to stimulate the vasopressin release are also known.

Table 4
The Diseases Most Often Associated with SIADH

1. Vasopressin-secreting malignant tumors
2. Nonmalignant pulmonary diseases: asthma, pneumonia, pulmonary tuberculosis, chronic obstructive pulmonary disease
3. Disorders of the central nervous system: meningitis, encephalitis, aneurysm, Guillain-Barré syndrome, trauma, brain tumors (nonvasopressin secreting)
4. Drugs: chlorpropamide, carbamazepin, vincristine, etc.

ESSENTIAL HYPERNATREMIA (CENTRAL HYPERNATREMIA OR HYPOTHALAMIC HYPERNATREMIA) The association of hypernatremia with neurologic lesions has been known for a long time. Particularly, lesions of the hypothalamus are likely to lead to the development of sustained hypernatremia and hyperosmolality as a consequence of specific disturbances in the neuroendocrine regulation of osmolality. In 1962, Welt suggested the use of the term "essential hypernatremia" to describe such patients [87]. The clinical features characteristic of the syndrome "essential hypernatremia" include: (1) chronic but fluctuating elevations of serum sodium level in the absence of decreased plasma volume; (2) impaired osmotic regulation of vasopressin secretion, although the endogenous production of vasopressin may be partially intact; and (3) hypernatremia that does not respond to chronic fluid overloading, but that may be corrected by vasopressin administration [88–90]. Thus, the sustained hyperosmolarity in patients with essential hypernatremia is the result of an elevated osmotic threshold for release of vasopressin. Because essential hypernatremia is caused by abnormalities in the control of the brain over the water–electrolyte metabolism, "central hypernatremia" or "hypothalamic hypernatremia" might be a more appropriate nomenclature for this disorder rather than "essential hypernatremia."

TUMORS AND CYSTIC LESIONS IN THE HYPOTHALAMUS AND NEUROHYPOPHYSIS

Various types of tumors and cystic lesions affect the hypothalamus and sellar region. The tumors include gliomas (Fig. 9), meningiomas, gangliocytomas, hamartomas, craniopharyngiomas (Fig. 12), germinomas, teratomas, and so forth. Hypothalamic hormone-secreting gangliocytomas and hamartomas were described in the earlier subheading, Hypothalamic Hormone–Secreting Tumors. In this section, other tumors and cystic lesions that specifically affect the hypothalamus are discussed. Rathke's

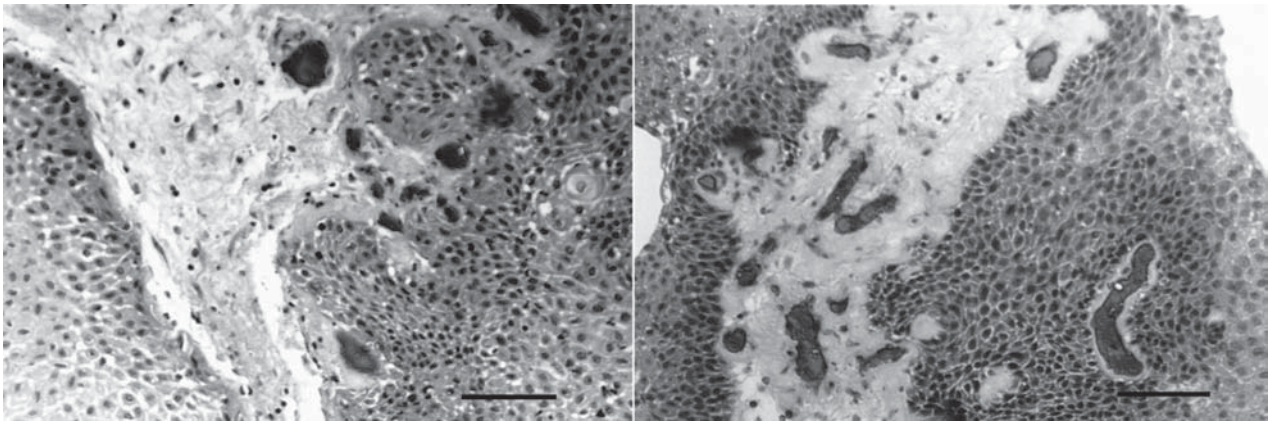

Figure 12 Craniopharyngioma (H&E). Bar = 50 μm. (Courtesy of Dr. H. Ikeda, Department of Neurosurgery, Tohoku University School of Medicine, Sendai, Japan.)

cleft cyst arises mainly intrasellarly, but often suprasellarly, and therefore is also described here.

CRANIOPHARYNGIOMA Craniopharyngioma is a cystic neoplasm derived from the remnants of Rathke's pouch (Fig. 12). It originates from the pituitary stalk and is usually suprasellar. Craniopharyngioma can be clinically aggressive with fingerlike infiltration to the surrounding structures, even if it is histologically benign. Craniopharyngioma may be seen at most ages, but it is most frequently diagnosed in childhood. It is the most common neoplasm associated with hypothalamo–pituitary dysfunction. Its clinical manifestations include visual dysfunction, anterior pituitary dysfunction, growth retardation, sexual dysfunction, diabetes insipidus, and cranial nerve abnormalities. Histologically, it is formed by complex cords or islands of squamous cells, and the outer layers of cells are usually cuboid to cylindrical. Craniopharyngioma has a cystic structure, which is formed by the lining-stratified squamous epithelium. Areas of mineralization or ossification are often found in the tumor tissue (Fig. 12). Calcification can be found on X-ray examination in the sellar or suprasellar region.

RATHKE'S CLEFT CYST Rathke's cleft cyst is a non-neoplastic, developmental sellar and/or suprasellar cystic lesion lined by a single layer of ciliated cuboidal or columnar epithelium (Figs. 13 and 14). It derives from a remnant of Rathke's pouch. Rathke's cleft cyst rarely comes symptomatic. When its size is enlarged by the accumulation of colloid secretion (more than 1 cm in diameter), symptoms and signs due to the compression by the cyst appear. These include hypopituitarism, hyperprolactinemia, diabetes insipidus, headache and impairment of visual acuity, and visual field defects [91]. A case of Rathke's cleft cyst with SIADH is also reported [92]. Approximately 50% of Rathke's cleft cysts are intrasellar, and the others are suprasellar, or suprasellar and intrasellar [93].

GERM CELL TUMORS Germ cell tumors include germinomas, choriocarcinomas, yolk sac tumors, embryonal carcinomas, teratomas with various degree of differentiation, and

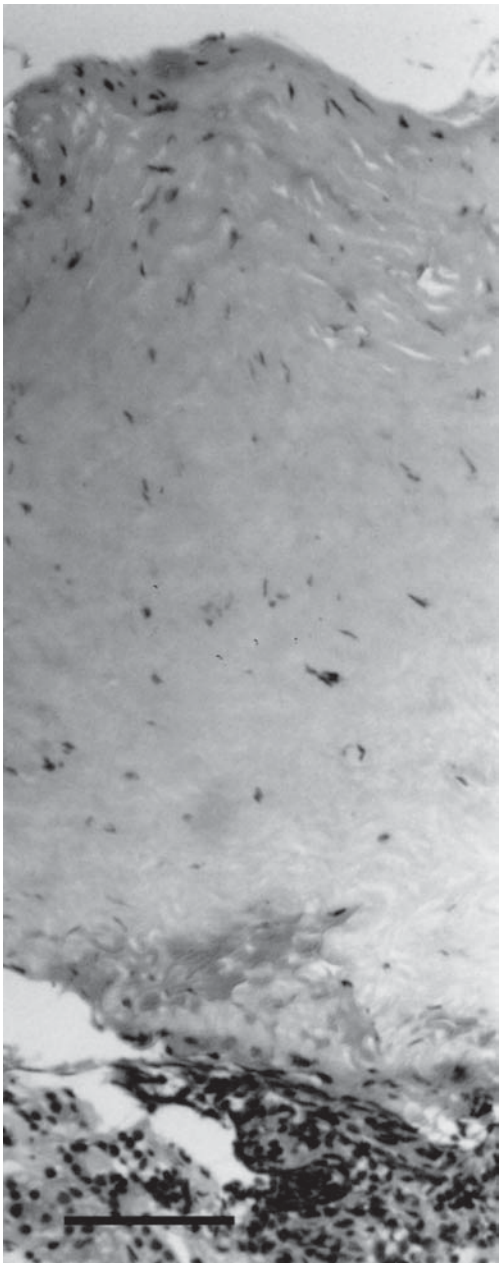

Figure 13 Cyst wall of Rathke's cleft cyst (H&E). Bar = 50 μm. (Courtesy of Dr. H. Ikeda, Department of Neurosurgery, Tohoku University School of Medicine, Sendai, Japan.)

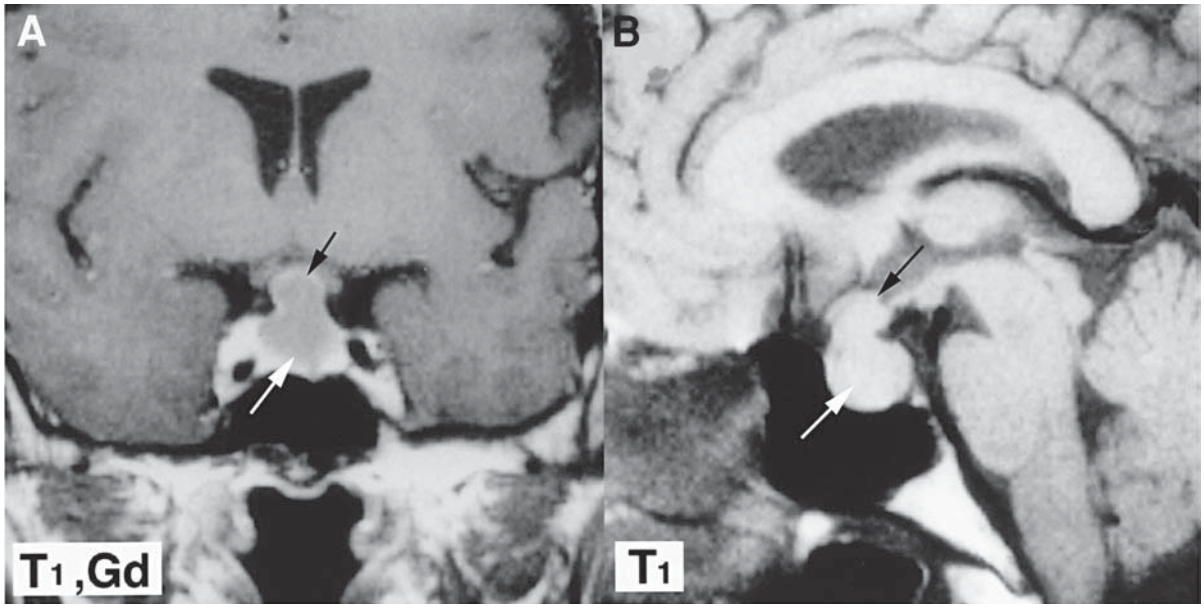

Figure 14 MRI of Rathke's cleft cysts (*arrows*). (**A**) Coronal T1-weighted MRI scan after gadolinium enhancement. (**B**) Sagittal T1-weighted MRI scan. The cyst is located in the suprasellar (shown by *black arrows*) and intrasellar (shown by *white arrows*) regions. (Reproduced from Takahashi K, Murakami O, Satoh F, Mouri T. The hypothalamus and neurohypophysis. In: Stefaneau L, Sasano H, Kovacs K, eds. Molecular and Cellular Endocrine Pathology. London: Arnold, 2000:45–74, with permission.)

mixed germ cell tumors. These tumors are presumed to originate from embryonic nests of germ cells, and are found in the gonads and extragonadally in the midline structures of the body. In the central nervous system, germ cell tumors arise in the pineal region and in the hypothalamohypophysial region. Among the germ cell tumors arising in the hypothalamohypophysial region, germinomas are the most common (suprasellar germinoma) [94]. Approximately 20% of germinomas produce human chorionic gonadotropin [95]. Clinical manifestations of suprasellar germinomas are diabetes insipidus, visual disturbance, and hypopituitarism (growth retardation or hypogonadism).

INFLAMMATION IN THE HYPOTHALAMUS AND NEUROHYPOPHYSIS

Inflammatory disease, such as Langerhans' cell histiocytosis and sarcoidosis, and infectious diseases (e.g., bacterial, viral, fungal, or tuberculous) can affect the hypothalamus and neurohypophysis.

LANGERHANS' CELL HISTIOCYTOSIS Langerhans' cell histiocytosis is a granulomatous disease that occurs mostly in children between 1 and 4 yr of age [96,97]. This disorder is characterized by the proliferation and infiltration of abnormal histiocytes within various tissues, which are morphologically and immunologically similar to Langerhans' cells, leading to the name Langerhans' cell histiocytosis. Chronic recurrent Langerhans' cell histiocytosis (Hand–Schüller–Christian disease) typically involves a triad of diabetes insipidus, proptosis, and destructive bone lesions, whereas the acute disseminated form of Langerhans' cell histiocytosis (Letterer–Siwe disease) is characterized by hepatosplenomegaly, fever, thrombocytopenia, anemia, and a rash. Eosinophilic granuloma is characterized by solitary bony disease. The key diagnostic feature of this disorder is the presence of abnormal aggregates of Langerhans' cells. These cells, unlike other histiocytes, are characterized by immunohistochemical positivity for CD1a and S-100 protein and by the ultrastructural presence of membranous cytoplasmic structures, 200–400 nm in width and shaped like tennis rackets, which are known as Birbeck granules [98,99].

Diabetes insipidus and growth retardation are the prominent endocrine manifestations of Langerhans' cell histiocytosis [99, 100]. Galactorrhea, hypogonadism, and panhypopituitarism are rarely associated with this disorder. Histiocytic infiltration results in a hypothalamic dysfunction with a secondary partial or complete hypopituitarism. This hypopituitarism is due to deficient trophic stimulation or inhibition by hypothalamic hormones or dopamine.

SARCOIDOSIS The hypothalamus and pituitary are the most commonly affected regions by sarcoidosis although endocrine manifestation is relatively rare in sarcoidosis [101–103]. The central nervous system is involved in about 5% of all cases of sarcoidosis, and diabetes insipidus occurs in 33% patients with neurosarcoidosis [104]. In addition to diabetes insipidus, patients with sarcoidosis often exhibit hypothalamic disturbances and anterior pituitary hormone deficiency [101–103]. Histologically, sarcoidosis shows noncaseous granulomatous tissue with multinucleated giant cells of foreign body type.

LYMPHOCYTIC INFUNDIBULONEUROHYPOPHYSITIS AND LYMPHOCYTIC HYPOPHYSITIS Lymphocytic infundibuloneurohypophysitis was proposed to be one major cause of what was previously considered to be idiopathic diabetes insipidus by Imura et al. [82]. They studied 17 patients with idiopathic diabetes insipidus. Magnetic resonance imaging (MRI)

showed that 9 of the 17 patients had thickening of the pituitary stalk, enlargement of the neurohypophysis, or both and lacked the hyperintense signal of the normal neurohypophysis. In the remaining eight patients, the pituitary stalk and the neurohypophysis were normal, although the hyperintense signal was absent. The abnormalities of thickening and enlargement were seen on MRI only in the patients who had diabetes insipidus for less than 2 yr, and the abnormalities disappeared during follow-up, suggesting that the natural course of the disorder is self-limited. In addition to vasopressin deficiency, two patients had mild hyperprolactinemia and nine had impaired secretory responses of growth hormone to insulin-induced hypoglycemia. The biopsies in two cases revealed chronic inflammation, with infiltration of lymphocytes (mainly T lymphocytes) and plasma cells.

On the other hand, lymphocytic hypophysitis is a rare inflammatory disease that primarily affects adenohypophysis and is probably caused by autoimmunity (Figs. 10 and 11). Approximately 19% of the patients with lymphocytic hypophysitis, however, have diabetes insipidus [83]. It predominantly affects women of menstrual age, in particular during late pregnancy or in the postpartum period. More than 70% of patients with this disease are female. Clinically, lymphocytic hypophysitis has an acute onset. Clinical manifestations are headaches, visual symptoms and signs, hypopituitarism, and radiological appearance of sellar mass lesion that mimics pituitary adenoma (Fig. 10). Lymphoplasmacytic infiltrate is a histological feature of this disease, as are occasional neutrophils, eosinophils, and macrophages in the anterior pituitary gland (Fig. 11). The inflammatory infiltrate was shown in the neurohypophysitis of some patients with lymphocytic hypophysitis and diabetes insipidus.

DIFFERENTIAL DIAGNOSIS OF MASS LESIONS THAT PRIMARILY INVOLVE THE POSTERIOR PITUITARY

Mass lesions that primarily involve the posterior pituitary are extremely uncommon. The differential diagnoses of neurohypophysial masses include neoplastic, infiltrative, or granulomatous diseases. Primary neoplasms originating from the posterior lobe are extremely rare; the most common neoplasms are granular cell tumors (choristomas or pituicytomas) [105]. The majority of such tumors remain asymptomatic and are found incidentally at autopsy. Secondary carcinomas involve the posterior pituitary more commonly than the anterior pituitary, and are usually found incidentally at autopsy in cases of disseminated carcinomatosis.

Infiltrative and granulomatous diseases are noted to have a predilection for the posterior pituitary. These include Langerhans' histiocytosis, sarcoidosis, tuberculosis, and syphilis, which can infiltrate the posterior lobe or pituitary stalk to cause diabetes insipidus [106].

HYPOGONADOTROPIC HYPOGONADISM AND KALLMANN'S SYNDROME

Hypogonadotropic hypogonadism is characterized by failed gonadal function secondary to deficient LH/FSH secretion and can result from lesions in the hypothalamo–pituitary–gonadal axis [107]. Isolated hypogonadotropic hypogonadism classically presents with delayed or absent puberty and has a prevalence of approx 0.025% in males and approx 0.01% in females. Approximately 50% of cases of isolated hypogonadotropic hypogonadism are accompanied with congenital anosmia and constitute Kallmann's syndrome. We describe here hypogonadotropic hypogonadism caused by abnormalities in the hypothalamus.

KALLMANN'S SYNDROME Kallmann's syndrome is characterized by the association of hypogonadism and inability to smell (anosmia). This syndrome is caused by a defect in the migration of olfactory neurons and neurons producing hypothalamic Gn-RH. Fetal Gn-RH neurosecretory neurons fail to migrate from the olfactory placode to the medial basal hypothalamus. The fetal Gn-RH-containing cells and neurites are arrested in their migration to the brain, and end in a tangle around the cribriform plate and in the dural layers adjacent to the meninges beneath the forebrain. Thus, hypogonadotropic hypogonadism is caused by the deficiency of hypothalamic Gn-RH, and the inability to smell by the absence of olfactory bulbs and tract. This syndrome is genetically heterogeneous and can be transmitted as an X-linked, autosomal dominant, or autosomal recessive trait. The *KAL1* gene, a candidate gene responsible for Kallmann's syndrome, was isolated from the critical region on Xp22.3, one of this syndrome's loci [108]. The gene product has significant similarities with proteins involved in neural cell adhesion and axonal path finding, suggesting that this gene product could have a specific role in neuronal migration.

OTHER HYPOGONADOTROPIC HYPOGONADISM RELATED TO THE HYPOTHALAMUS Familial isolated hypogonadotropic hypogonadism is also caused by other genetic conditions related to the hypothalamus. Mutations in the Gn-RH receptor gene cause gonadotroph resistance to Gn-RH stimulation and result in hypogonadotropic hypogonadism [34,35]. Hypogonadism in the mutant *hpg* mouse is characterized by a deficiency of hypothalamic Gn-RH [109]. Human cases of Gn-RH deficiency due to Gn-RH gene mutations, however, have not been reported [110].

Patients with hypogonadotropic hypogonadism are frequently accompanied by obesity. These include Bardet–Biedl syndrome, Prader–Willi syndrome, leptin gene mutation [111,112], leptin receptor gene mutation [113], and prohormone convertase 1 gene mutation [114,115]. Fröhlich's syndrome, which is caused by the destruction of the ventromedial nucleus of hypothalamus by an invasive tumor, also have both obesity and hypogonadotropic hypogonadism. We describe these diseases in the later subheading, Obesity. Leptin not only suppresses the appetite, but also appears to activate the hypothalamo–pituitary gonadal axis. For example, the normal rise of testosterone at the onset of puberty in young boys is preceded by a peak of leptin secretion [116]. Moreover, leptin stimulates the secretion of Gn-RH by hypothalamic neurons and gonadotropins by pituitary cells in vitro [117]. Hypogonadotropic hypogonadism is therefore caused by leptin gene mutation [111,112] or leptin receptor gene mutation [113]. The other diseases with hypogonadotropic hypogonadism and obesity appear to be due to the primary hypothalamic dysfunction. Hypogonadotropic hypogonadism in patients with prohormone convertase 1 gene muta-

Table 5
Representative Neuropeptides, Neurotransmitters, and Hormones that Influence Eating Behavior

Stimulate eating	*Inhibit eating*
NPY	Leptin[b]
Melanin-concentrating hormone	α-MSH
Agouti-related protein	CRH/urocortin
Orexins (hypocretins)	TRH
Ghrelin[a]	Cocaine- and amphetamine-regulated transcript peptide
	Peptide YY(3-36)[c]
Galanin	CGRP
Opioids	Insulin
α_2-Noradrenergic	Prolactin-releasing peptide
GABA	Somatostatin
GH-RH	Cholecystokinin
Opioid peptides	Glucagon-like peptide-1 and -2
	Neurotensin

[a]Secreted mainly from stomach and acts on the hypothalamus.
[b]Secreted mainly from adipocytes and acts on the hypothalamus.
[c]Secreted mainly from the gastrointestinal tract and acts on the hypothalamus.

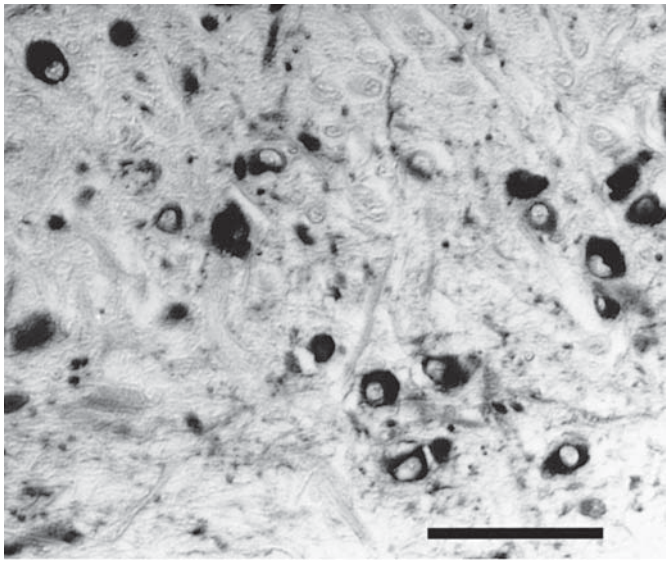

Figure 15 NPY in the human hypothalamus. NPY immunoreactive cell bodies are localized in the infundibular nucleus. Bar = 50 μm.

tions may arise from impaired processing of hypothalamic hormones including Gn-RH and neuropeptides related to Gn-RH secretion [115].

APPETITE REGULATION, OBESITY, AND ANOREXIA NERVOSA

Disruption of the ventromedial hypothalamus produced hyperphagic obesity, while lesions of the lateral hypothalamus caused hypophagia and weight loss. These observations suggest the existence of ventromedial "satiety" and lateral "feeding" centers. Since the recent discovery of leptin, a peptide hormone derived from adipocytes, studies on the central regulation of appetite and obesity have been greatly advanced.

Leptin is a 167-amino-acid peptide that is secreted from adipocytes; acts on the brain, particularly the hypothalamus; and suppresses the appetite and food intake, and increases metabolic activity [12,118]. Leptin was originally described as the product of the mouse *obese (ob)* gene [12]. In *ob/ob* mice, obesity was caused by mutation of the *ob* gene, which results in a lack of circulating leptin. In contrast, the mutation of the leptin receptor gene causes obesity in *db/db* mice. Plasma levels of leptin are elevated in obese subjects, whereas they are low in lean subjects.

A number of neurotransmitters and neuromodulators, mostly neuropeptides, have been demonstrated to regulate the appetite in the hypothalamus (Table 5). Leptin secreted by adipocytes is supposed to regulate the appetite by affecting the production and secretion of these neuropeptides in the hypothalamus. Representative appetite-stimulating factors are neuropeptide Y (NPY) (Fig. 15) and melanin-concentrating hormone (MCH) (Fig. 16).

NPY is a 36-amino-acid peptide originally isolated from porcine brain, and together with PP and peptide YY (PYY), forms the pancreatic polypeptide (PP) peptide family [119]. In peripheral tissues, NPY is localized in the sympathetic nerves, adrenal medulla, and so forth, and is one of the most potent vasoconstrictor peptides. NPY is the most abundantly expressed neuropeptide in the brain. In the human hypothalamus, NPY is localized in the infundibular nucleus (Fig. 15) and paraventricular nucleus [120]. NPY in the infundibular nucleus has been shown to act as a potent appetite stimulator [121].

MCH was originally isolated from salmon pituitary as a skin-color-regulating hormone [122]. MCH has an antagonistic action against α-melanocyte-stimulating hormone (α-MSH), and make the skin color white by aggregating melanosome within melanophores in fishes. MCH is expressed predominantly in the hypothalamus of mammals and acts as a neurotransmitter or a neuromodulator [123,124]. MCH-containing perikarya are found in the posterior and lateral hypothalamic areas, particularly around the mamillary body and fornix (posterior nucleus and perifornical nucleus) (Fig. 16). MCH expression was increased in obese mice (*ob/ob* mice) and the central injection of MCH increased the feeding in rats [125]. Recent studies have shown that MCH knockout mice had reduced body weight [126], supporting a role of MCH as a appetite stimulator. There are at least two subtypes of MCH receptors, MCH receptors 1 and 2, which are distinct from the melanocortin receptors (receptors for ACTH and/or α-MSH) [127–129]. Other appetite-stimulating hormone, orexins (hypocretins) [130,131], are also expressed in similar areas of hypothalamus to MCH, but rarely colocalize with MCH in the same neurons [132].

α-MSH is generated from pro-opiomelanocortin (POMC), the common precursor protein of ACTH and endorphins, by post-translational enzymatic proteolytic processing. α-MSH corresponds to the N-terminal 1–13 portion of ACTH. The POMC neurons are present in the infundibular nucleus of hypothalamus. α-MSH expressed in this nucleus acts as a potent inhibitor of appetite [133]. There are at least five subtypes of melanocortin receptors. The melanocortin 1 receptor mediates an action of α-MSH on melanocytes, and the melanocortin 2 receptor

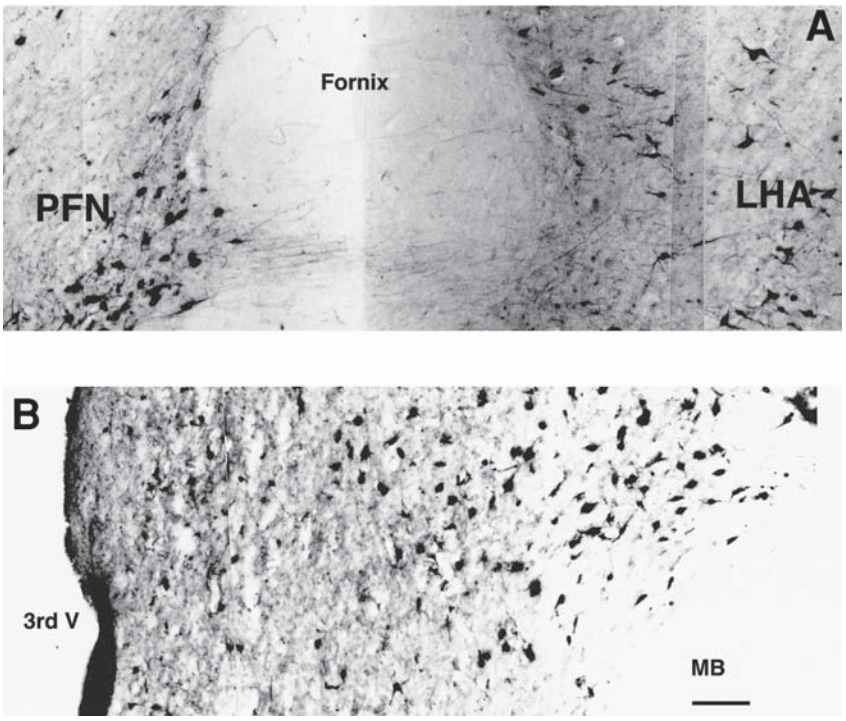

Figure 16 MCH in the human hypothalamus. MCH immunoreactive cell bodies are exclusively localized in the posterior and lateral hypothalamic areas (LHA), including perifornical nucleus (PFN) (**A**) and posterior nucleus (**B**). 3rd V, Third ventricle; MB, mammillary body. Several photographs are combined. MCH-immunoreactive nerve fibers derived from these hypothalamic neurons are distributed throughout in the brain and pituitary. Bar = 200 μm.

mediates an action of ACTH on the adrenal cortex to produce and secrete glucocorticoids. The melanocortin 3 and 4 receptors mediate suppressive actions of α-MSH on appetite in the brain, particularly in the hypothalamus. Agouti protein is expressed in the mouse skin and regulates coat color by binding to and antagonizing the melanocortin 1 receptor. Agouti-related protein is a 132-amino-acid protein with 25% homology to the Agouti protein [134]. Agouti-related protein is expressed in the infundibular nucleus of the hypothalamus and stimulates the appetite by antagonizing the actions of α-MSH on the melanocortin 3 and 4 receptors.

Ghrelin is secreted from the stomach and hypothalamus [25]. In addition to the GH release activity, ghrelin has appetite-stimulating actions. It has been shown that both intracerebroventricular and intraperitoneal administration of ghrelin in rats stimulates food intake [135,136]. Thus, not only ghrelin of the hypothalamic source, but also ghrelin secreted from the stomach, are supposed to act on the hypothalamus and stimulate appetite. Plasma concentrations of ghrelin are high during fasting whereas they fall to a nadir within an hour of eating [137], suggesting a role of ghrelin in meal initiation.

The schematic representation of the putative relationship among leptin, ghrelin, and appetite-stimulating and inhibiting neuropeptides in the hypothalamus is shown in Fig. 17. Leptin receptor is expressed in the arcuate nucleus (infundibular nucleus), where leptin appears to regulate the secretion of appetite-stimulating and inhibiting neuropeptides, such as NPY and α-MSH.

GENETIC DISEASES ASSOCIATED WITH OBESITY RELATED TO THE HYPOTHALAMUS Obesity is caused by many environmental factors and genetic predisposition in most cases. Recent studies have shown that certain types of obesity are caused by mutations in the single gene encoding the hormone, the hormone receptor, or the processing enzyme, which are related to appetite regulation in the hypothalamus (Table 6). It should be noted, however, that such cases are very rare. On the other hand, polymorphism in these genes may be related to the pathogenesis of obesity in many more cases.

Obesity Due to Single-Gene Mutations

Mutations in the Gene of Leptin or Leptin Receptor Montague et al. [111] reported two severely obese children who are members of the same highly consanguineous pedigree in Pakistan. Their serum leptin levels were very low despite their markedly elevated fat mass. A homozygous frameshift mutation involving the deletion of a single guanine nucleotide in codon 133 of the gene for leptin was found in both subjects. Strobel et al. reported that sequencing of the leptin gene from a Turkish obese patient with a low serum leptin level uncovered a missense mutation in codon 105, which leads to the substitution of an Arg for Trp at position 84 of the mature protein [112]. Although a normal size leptin protein is synthesized in this subject, this is not secreted in serum. Patients with leptin deficiency showed multiple endocrine defects, decreased sympathetic tone, and immune system dysfunction [138]. The endocrine defects include hypogonadism, impaired renin–aldosterone function, and alternations in GH and PTH–calcium function.

Clement et al. reported a homozygous mutation in the human leptin receptor gene that results in a truncated leptin receptor lacking both the transmembrane and the intracellular domains

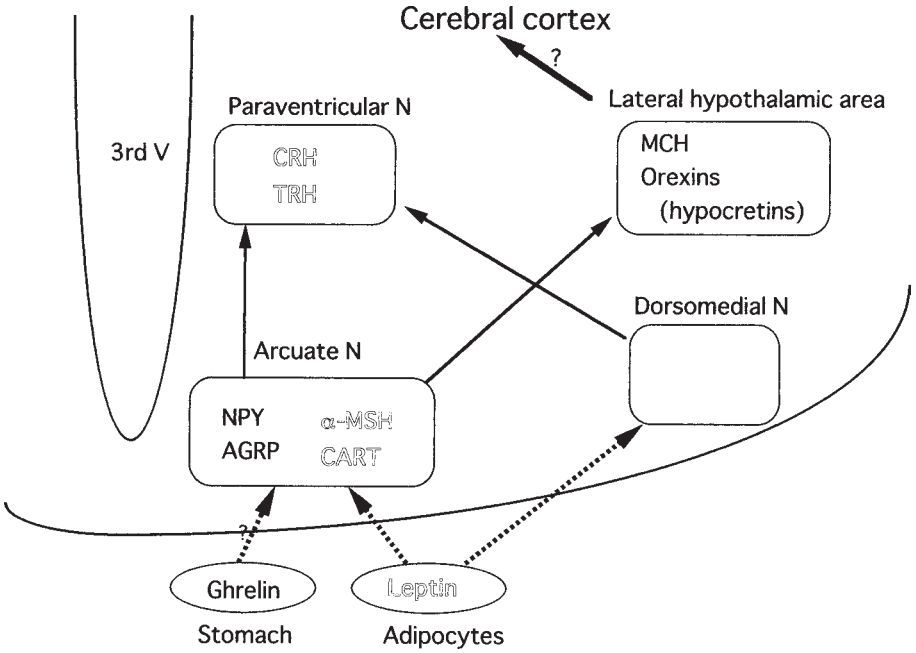

Figure 17 Schematic representation of the putative relationship among leptin, ghrelin, and appetite-stimulating and inhibiting neuropeptides in the hypothalamus. Appetite-inhibiting factors are shown in *outlined* characters.

Table 6
Obesity Caused by Single Gene Mutations in Humans

Genes	*Mechanism causing obesity*	*Refs.*
Melanocortin-4 receptor	Deficiency of α-MSH action to suppress appetite in the hypothalamus	141–143
Melanocortin-3 receptor	Deficiency of α-MSH action to suppress appetite in the hypothalamus	144
POMC	Deficiency of α-MSH secretion in the infundibular nucleus of the hypothalamus	139
Leptin	Deficiency of functioning leptin secretion by adipocytes	111,112
Leptin receptor	Deficiency of leptin action to suppress appetite in the hypothalamus	113
Prohormone convertase 1	Deficiency of α-MSH processing in the infundibular nucleus of hypothalamus and insulin processing in the pancreatic β-cells (?)	114,115

[113]. In addition to their early-onset morbid obesity, patients homozygous for this mutation have no pubertal development and their secretion of growth hormone and thyrotropin is reduced.

Mutations in the* POMC *Gene or Melanocortin Receptors Krude et al. reported cases of a genetic defect within the *POMC* gene that showed early-onset obesity, adrenal insufficiency, and red hair pigmentation [139]. Patient 1 was found to be a compound heterozygote for two mutations in exon 3 (G7013T, C7133δ), which interfere with appropriate synthesis of ACTH and α-MSH. Patient 2 was homozygous for a mutation in exon 2 (C3804A) which abolishes *POMC* translation. α-MSH has a dual role in regulating food intake in the hypothalamus and influencing hair pigmentation, and therefore the deficiency of α-MSH may result in obesity and red hair pigmentation. The deficiency in ACTH may result in secondary adrenal insufficiency. *POMC* gene knockout mice lacking the *POMC*-derived peptides have obesity, defective adrenal development, and altered pigmentation, a phenotype similar to that of the human *POMC*-deficient patients [140].

The melanocortin-3 and -4 receptors mediate the suppressive actions of α-MSH on appetite in the hypothalamus. Vaisse et al. reported two families with frameshift mutations in melanocortin-4 receptor gene that caused an early-onset dominant form of obesity [141]. Subsequent studies have shown that melanocortin-4 receptor gene mutations are a frequent but heterogeneous genetic cause of morbid obesity [142,143]. Thus, melanocortin-4 receptor gene mutations may be the most frequent causes for obesity among patients with obesity due to single-gene mutations. A mutation (*Ile183Asn*) in the melanocortin-3 receptor gene was also found in obese subjects [144].

Mutations in the Prohormone Convertase 1 Gene Prohormone convertase 1 is an endopeptidase that is expressed in neuroendocrine tissues and processes the prohormones, including proinsulin and POMC. A patient with prohormone convertase 1 deficiency showed an extreme obesity, abnormal glucose homeostasis, hypogonadotropic hypogonadism, hypocorticoidism, and elevated plasma proinsulin and POMC concentrations but very low plasma levels of insulin and ACTH [114]. Analysis of prohormone convertase 1 gene of this patient showed that this proband is a compound heterozygote for mutations in the prohormone convertase 1 gene [115].

Other Genetic Diseases Associated with Obesity

Bardet–Biedle Syndrome Bardet–Biedl syndrome is an autosomal recessive disorder that is characterized by retinitis

pigmentosa, polydactyly, obesity, mental retardation, hypogonadism, renal dysplasia, and short stature [145]. This disorder is heterogeneous and at least four gene loci responsible for this disorder have been mapped:11q13 (*BBS1*), 16q21 (*BBS2*), 3p12 (*BBS3*), and 15q22 (*BBS4*). Laurence–Moon syndrome and Bardet–Biedle syndrome are now regarded as distinct entities [146]. Like Bardet–Biedle syndrome, Laurence–Moon syndrome is an autosomal recessive disorder that is characterized by retinitis pigmentosa, hypogonadism, and developmental delay. Laurence–Moon syndrome, however, is associated with spastic paraplegia.

Prader–Willi Syndrome Prader–Willi syndrome is a genetic disorder characterized by a range of mental and physical symptoms [147]. These include short statue, muscular hypotonia, excessive appetite with progressive obesity, hypogonadism, mental retardation, behavioral abnormalities, sleep disturbances (including sleep apnea), and dysmorphic features. It is estimated that one child in every 10,000–25,000 live births suffers from this syndrome. Occurring in 70–75% of affected individuals, the principal genetic mutation associated with the condition is deletion of a segment of the paternally derived chromosome 15 (15q11–q13). Several other abnormalities have also been linked with the syndrome: 20–25% of patients exhibit maternal disomy of the same region of chromosome 15, 2–5% have imprinting center mutations, and 1% have translocations. The individual gene or genes from within 15q11–q13 that cause the condition have yet to be identified.

Reduced GH secretion and hypogonadotropic hypogonadism occur in the majority of patients with Prader–Willi syndrome, together with abnormal appetite control and high pain threshold. This suggests that patients with Prader–Willi syndrome have hypothalamic–pituitary dysfunction. Autopsies of five patients with Prader–Willi syndrome showed that the paraventricular nucleus was reduced in size and there were fewer oxytocin-expressing neurons [148]. There were a 30% reduction in GH-RH-releasing neurons in the arcuate nucleus, a downregulation of NPY, and a deficiency in vasopressin [149]. MRI has revealed a complete absence or a small size of the bright spot in the posterior pituitary lobe of four of 15 affected individuals, which is considered to be a sign of hypothalamic dysfunction [150].

OBESITY DUE TO NONGENETIC HYPOTHALAMIC CAUSES

Fröhlich's Syndrome Fröhlich's syndrome (adiposogenial dystrophy) was originally characterized as delayed puberty, hypogonadism, and obesity associated with a tumor that impinges on the hypothalamus [151]. Several organic lesions of the hypothalamus, however, can cause this disorder, including tumors, encephalitis, microcephaly, Friedreich's ataxia, and demyelinating diseases.

ANOREXIA NERVOSA Anorexia nervosa is a functional disorder characterized by refusal to maintain body weight at or above a minimally normal weight for age and height, intense fear of gaining weight, body image disturbance, and amenorrhea [152]. The etiology of this disorder is unknown. It occurs most often in young women. The following multiple endocrine disturbances and hypothalamic dysfunction are known to occur in patients with anorexia nervosa. Urinary and plasma levels of gonadotropins are low. Plasma cortisol levels and cerebrospinal fluid levels of CRH are elevated. This may be consistent with results in animal experiments showing that central administration of CRH decreased feeding and LH secretion. Plasma GH levels are elevated, whereas plasma levels of insulin-like growth factor 1 are decreased. Plasma levels of leptin are reduced, with low weight and percentage body fat in subjects with anorexia nervosa [153]. It has been shown that leptin has a stimulatory action on the hypothalamo–pituitary–gonadal axis [154], raising the possibility that hypogonadism associated with anorexia nervosa may partly be due to the leptin deficiency. On the other hand, the hypothalamic dysfunction with multiple endocrine disturbances seen in anorexia nervosa may be a secondary phenomena due to the unknown central disturbance.

NARCOLEPSY AND OREXINS (HYPOCRETINS)

Narcolepsy is a disabling neurological disorder that affects more than 1 in 2000 individuals. This disorder is characterized by daytime sleepiness; sleep fragmentation; and symptoms of abnormal REM sleep, such as cataplexy, sleep paralysis, and hypnagogic hallucinations [155]. Most human cases of narcolepsy occur sporadically and the disorder is generally believed to be multigenic and environmentally influenced, whereas in canines (Doberman pinschers) the disorder is transmitted as a single autosomal recessive trait. One predisposing genetic factor in human narcolepsy is a specific *HLA-DQ* allele, *HLA-DQB1*0602*. Because of the close HLA association, the human narcolepsy was suggested to be autoimmune in nature. Recent studies have shown that the neuropeptides orexins (hypocretins) are involved in the pathogenesis of narcolepsy [155–158].

The orexins consist of two peptides: orexin-A, a 33-amino-acid peptide, and orexin-B, a 28-amino-acid peptide, which are derived from the same precursor by proteolytic processing [130,131]. The orexins are named following their central appetite-stimulating action. These peptides are specifically expressed in the hypothalamus, and the positive cell bodies are restricted to the lateral and posterior hypothalamus. The actions of orexins are mediated by two G-protein-coupled receptors named orexin-1 receptor and orexin-2 receptor.

Canine narcolepsy has been shown to be caused by a mutation in the orexin receptor 2 gene [155]. Transgenic mice with ablation of orexin-containing neurons showed a phenotype strikingly similar to human narcolepsy, including behavioral arrests, premature entry into REM sleep, and poorly consolidated sleep patterns [156]. In most human cases of narcolepsy, orexin levels in the cerebrospinal fluid have been undetectable [157]. Studies using postmortem brain obtained from patients with narcolepsy showed loss of orexins in the posterior and lateral hypothalamic areas, without gliosis or signs of inflammation [158]. On the other hand, one orexin mutation, impairing peptide trafficking and processing, was found in a single case with early onset narcolepsy among 74 patients. Although orexin loci do not contribute significantly to genetic predisposition, most cases of narcolepsy are associated with a deficient orexin system.

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3 Tumors of the Adenohypophysis

SERGIO VIDAL, PHD, EVA HORVATH, PHD,
KALMAN KOVACS, MD, PHD, AND BERND W. SCHEITHAUER, MD

INTRODUCTION

The human pituitary is an oval, bean-shaped, and bilaterally symmetric organ located in the sella turcica, near the hypothalamus and optic chiasm, surrounded by the sphenoid bone, and covered with the sellar diaphragm. It is a composite endocrine organ divided in two parts: the adenohypophysis, which derives from an evagination of stomodeal ectoderm (Rathke pouch), and the neurohypophysis, which arises from the neuroectoderm of the floor of the forebrain. The adult pituitary weighs about 0.6 g and measures about 13 mm transversely, 9 mm anteroposteriorly, and 6 mm vertically. A reduction in weight is evident in old age, and an increase occurs during pregnancy and lactation. Although the pituitary size regresses after cessation of lactation, the reversion is not complete, the gland weighing 1 g or more in multiparous women.

The adenohypophysis (anterior lobe) comprises approx 80% of the entire pituitary and includes the pars distalis (PD), the pars intermedia (PI), and the pars tuberalis (PT). It produces six distinct hormones, including the three amino acid hormones—growth hormone (GH), prolactin (PRL), and adrenocorticotropin (ACTH)—as well as the three glycoprotein hormones—thyrotropin or thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH), and luteinizing hormone (LH). Collectively, these affect the function of virtually all cells in the body [1]. Our perceptions of the functional anatomy and cytology of the human pituitary gland underwent considerable change in the last three decades of the 20th century. The enduring concept of the human adenohypophysis comprising five cell types irreversibly committed to produce six hormones gradually gave way to a new paradigm. In the course of work aimed at developing a morphological classification of pituitary adenomas, three distinct tumor types not related to any of the five known cell types were recognized. This finding suggested the existence of three previously unrecognized cell types. Two of these appeared to produce pro-opiomelanocortin (POMC), the prohormone shared by anterior lobe corticotrophs and cells of the PI, thus challenging the assumption that the human PI is vestigial and lacks functional significance. Subsequently, it was demonstrated that, following normal embryonic growth, the PI migrates into the anterior lobe during the late fetal period. In later life the PI-derived, POMC-producing cells may proliferate, giving rise to either type of silent "corticotroph" adenoma [2]. To date, the parent cell of this third, newly recognized tumor type has eluded detection. A minor component of the adenohypophysis, the PT, surrounds the anterolateral aspect of the pituitary stalk and is assumed to play only a minor role in adenohypophysial function. Hormone secretion by the adenohypophysis is regulated primarily by hypothalamic stimulating and inhibiting hormones, which, synthesized in various nuclei of the hypothalamus, are transported via the portal vessels to adenohypophysial cells. Recent evidence indicates that the regulation of adenohypophysial hormone secretion is more complicated than previously thought. Peripheral, target organ gland hormones exert a powerful feedback effect, not only on the hypothalamus but also directly on adenohypophysial cells. In addition, several growth factors and cytokines affect hormone secretion. One novel finding is that several growth factors as well as hypothalamic hormones are produced by adenohypophysial cells as well. Via paracrine/autocrine effects, these substances can modulate adenohypophysial hormone release.

As a downward extension of the hypothalamus, the neurohypophysis or pars nervosa consists of three portions, including the median eminence, the hypophysial stalk or "infundibulum," and the pars posterior or posterior lobe. The latter plays an important role in the secretion of vasopressin and oxytocin, and consists of terminations of nerve fibers arising in the supraoptic and paraventricular nuclei of the hypothalamus. These nerve endings contain neurosecretory granules and are surrounded by specialized glial cells termed pituicytes. The posterior pituitary hormones, synthesized in the supraoptic and paraventricular nuclei of the hypothalamus, are bound to carrier proteins (neurophysins) and are transported via the unmyelinated nerve fibers to the posterior lobe, where they are stored in the neurosecretory granules until subsequently released [3].

Given the complex anatomy of the sellar region, as well as the crucial role of the pituitary in regulating the body's hormonal balance, the clinical manifestations of its diseases are highly variable. It is assumed that some lesions exhibit primarily endocrine effects, whereas others produce mechanical, compressive effects on critical structures surrounding the gland. In

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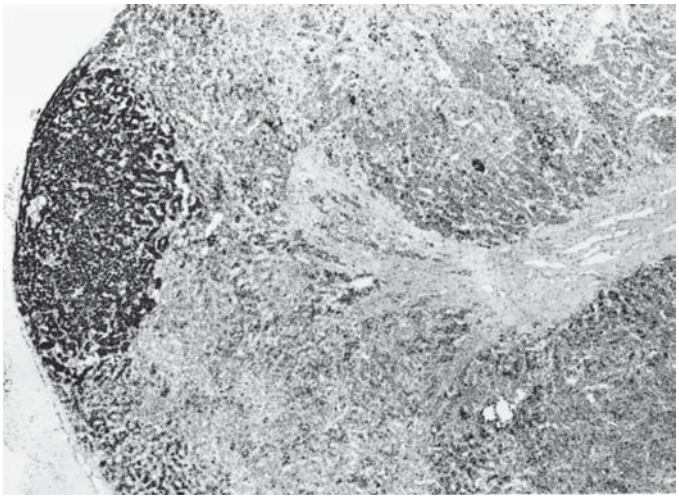

Figure 1 Incidental microadenomas are a frequent autopsy finding in the elderly. They are benign, well-demarcated tumors, which either lack imunoreactivity for pituitary hormones or stain mainly for prolactin as is shown in the picture. Original magnification ×40.

numerical and clinical terms, pituitary tumors are the most significant lesions affecting the sellar region. Although these may affect either the adenohypophysis or neurohypophysis, nearly all originate in the former. Neurohypophysial tumors are not only rare but also show far less diversity. The neurohypophysis is, however, a favored recipient site of various metastatic tumors.

Pituitary tumors consisting of adenohypophysial cells represent a unique form of neoplasia. In concept and practice, they differ from virtually all other tumors affecting the sellar region, for example, meningeal, neural, glial, vascular, osseous, and embryonal neoplasms. Of these, some clinically and radiographically mimic pituitary adenoma, thus making a firm preoperative distinction impossible. Also entering into the differential diagnosis of adenoma are various non-neoplastic, "tumor-like" lesions.

The primary focus of this chapter is a review of our current knowledge of adenohypophysial tumors and a discussion of their differential diagnosis.

TUMORS OF THE ADENOHYPOPHYSIS

Tumors of the adenohypophysis are not only the principal tumors of the sellar region, but with the possible exception of meningiomas, also the most frequent primary intracranial neoplasms seen in clinical practice. They represent approx 10–15% of all operated intracranial tumors and are encountered in 20–25% of autopsy-obtained pituitaries. Thus, neoplastic transformation in the pituitary is a relatively common event but one not always manifesting clinically (Fig. 1).

Although no age group is exempt from the development of adenomas, there is a clear tendency for their frequency to increase with age, the highest incidence being between the third and the sixth decades. They are only rarely diagnosed in prepubertal patients. On the basis of surgical series, pituitary tumors occur more often among women, particularly prolactin cell adenomas in premenopausal women. The basis of their prevalence in women is unclear, especially given the fact that in autopsy series incidental adenomas are equally distributed between the two sexes. The expression of estrogen and other sex steroid receptors in the normal pituitary may in part account for the female preponderance. Other factors may also be involved in that clinical manifestation is more conspicuous and easily recognized in women [4].

The overwhelming majority of neoplastic lesions arising in the adenohypophysis are adenomas. Nearly all are histologically benign, slow-growing, well-demarcated, and confined to the sella turcica. In other cases, however, they exhibit rapid proliferation and are invasive of dura, bone, and vascular adventitia. Invasion of these structures is indicative of malignancy. Pituitary carcinoma is exceedingly rare and is defined as a metastasizing tumor giving rise to cerebrospinal and/or distant systemic metastases [5–8]. Brain invasion, although less well understood, is also considered a sign of malignancy.

Based on their remarkable variation in biological behavior, numerous attempts have been made to classify pituitary adenomas into distinct categories. In the present chapter, we present the five-tier classification scheme of pituitary tumors now embodied in the *World Health Organization International Histological Classification of Tumors* [9,10]. This approach takes into consideration the clinical and laboratory findings, neuroimaging findings, as well as histologic, immunocytochemical, and ultrastructural features.

CLASSIFICATION OF PITUITARY TUMORS BASED ON CLINICAL FINDINGS AND ENDOCRINE DATA Although these parameters are clinical and biochemical, in most cases, they correlate with tumor morphology and immunohistochemistry. As such, they are valuable to diagnostic pathologists [1,11]. Ordinarily, the history and physical examination provide important indications as to the endocrine status of the patients. Suspicions of hormone excess and/or deficiency must then be validated by careful endocrine testing. An endocrine diagnosis is reached by measuring pituitary and target gland hormone levels in both basal and dynamic states. Such measurements are sensitive diagnostic indicators in the approx 70% of pituitary tumors that are hormonally active. The remainder are functionally "silent" and present as expanding sellar masses that cause panhypopituitarism or nonendocrine symptoms due to compression of other anatomic structures in the sellar region. Thus, a variety of clinical features, in either isolation or combination, can be associated with pituitary tumors.

Endocrinologically functioning adenomas cause pituitary hormone excess and a variety of distinctive hypersecretory states. These include hypersecretion of GH, PRL, ACTH, and, rarely, TSH. Corresponding clinical phenotypes include acromegaly or gigantism, the amenorrhea–galactorrhea syndrome, Cushing's disease/Nelson's syndrome, and hyperthyroidism. Clinically nonfunctioning pituitary adenomas, mainly gonadotrophic or null cell adenomas, present as expanding sellar masses. Owing to compression or injury to the nontumorous pituitary, its stalk, or the hypothalamus, they are often associated with various degrees of hypopituitarism.

The clinical presentation of both functioning and nonfunctioning pituitary adenomas may include a constellation of neurologic symptoms. Suprasellar extension, with compression of

the optic chiasm, results in a characteristic bitemporal hemianoptic pattern of visual loss. Encroachment on hypothalamic structures causes alterations in the sleep cycle, alertness, and behavior. Occasional transgression of the lamina terminalis brings pituitary adenomas into the region of the third ventricle with resultant obstructive hydrocephalus. Lateral extension of pituitary adenomas with entry into one or both cavernous sinuses occurs quite commonly and produces cranial neuropathies (cavernous sinus syndrome). Some tumors extend in other directions and, if sufficiently large, can involve the anterior, the middle, and occasionally the posterior fossae, wherein they can produce a full spectrum of neurologic deficits. Common symptoms of large pituitary tumors include headache and increased intracranial pressure.

As noted above, an important effect of a large pituitary tumor is the development of hypopituitarism. Although its causes are varied, it is usually the result of compression or destruction of the hypothalamus and/or pituitary stalk. As the hypothalamus plays a major role in regulating pituitary secretory activity, hypopituitarism may be of hypothalamic origin, that is, the result of decreased or absent secretion of regulatory hypothalamic hormones. Another important presentation is the so-called "stalk-section effect" wherein anterior lobe dysfunction (hyperprolactinemia due to cessation of dopamine delivery to lactotrophs) and diabetes insipidus are principal effects.

Stimulatory hormones such as growth hormone-releasing hormone (GH-RH), corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH), and gonadotropin-releasing hormone (GnRH) as well as inhibitory hormones, such as dopamine (DA) or somatostatin (SST), are synthesized by parvocellular neurons of the hypothalamus. They are often released from axon terminals in the external zone of the median eminence into hypophysial portal vessels. The pituitary gland has a unique blood supply originating in the inferior and superior hypophysial arteries, both branches of the internal carotid arteries [12]. The inferior hypophysial arteries transport arterial blood directly to the pituitary capsule, a few rows of adenohypophysial cells under the capsule, and the neurohypophysis. The superior hypophysial arteries are divided into two main branches; of these, one penetrates the infundibulum and terminates in the surrounding capillary network, whereas the other, a descending branch known as the loral artery, provides direct arterial blood supply to the anterior lobe without passing through the infundibulum. From the functional point of view, the capillary network surrounding the infundibulum plays a crucial role in the regulation of adenohypophysial endocrine activity. From hypothalamic nerve endings, the releasing and inhibitory hormones pass into the capillary network. Deriving from this network, the long portal vessels extend down the hypophysial stalk to terminate in adenohypophysial capillaries where the hormones gain ready access to the various secretory cells. Short portal vessels originating in the distal stalk and posterior lobe also enter the adenohypophysis.

Compression of the pituitary stalk not only results in disruption of the blood flow to the hypophysial portal system but also impairs transit of vasopressin and oxytocin via nerve fibers to the posterior lobe. Because the normal functional activity of the posterior lobe depends upon the integrity of its nerve fiber tracts, disruption of this pathway results in diabetes insipidus (*see* below).

Although the clinical manifestations of hypopituitarism are influenced by its etiology, severity, and rate of development, a characteristic evolution of pituitary failure is apparent. Secretion of LH and FSH are usually affected first, followed sequentially by TSH and GH. The ACTH axis is most resilient and is generally the last to be affected. Prolactin deficiency is rare except as a component of Sheehan's syndrome (postpartum pituitary necrosis). In contrast, hyperprolactinemia is far more common, and due to loss of production or effective transport and release dopamine, the hypothalamic prolactin-inhibiting hormone. Moderate hyperprolactinemia (±100 ng/mL) can occur in association with any structural lesion of the sellar region. Thus, its presence should not prompt a reflex diagnosis of PRL-producing adenoma. This is actually related to the fact that PRL secretion is under the inhibitory control of various hypothalamic "prolactin inhibitory factors," of which dopamine is the most important. Processes that impair the hypothalamic release of dopamine (compressive or destructive hypothalamic lesions), or impair its adenohypophysial transfer (compressive or destructive lesions of the pituitary stalk), disinhibit PRL cells with resultant hyperprolactinemia. Also an important symptom associated with hypopituitarism is diabetes insipidus, a result of the diminished functional activity of the posterior lobe. It is intriguing that diabetes insipidus practically never occurs in patients with pituitary adenomas.

Although the most common cause of hypopituitarism is pituitary adenoma, other potential causes should be considered. These include: nonpituitary neoplasms of the sellar region, for example, craniopharyngiomas; metastases to the pituitary from carcinomas of the breast, lung, colon, and prostate; vascular disorders, for example, pituitary apoplexy; inflammatory lesions, for example, lymphocytic hypophysitis, giant cell granuloma, sarcoidosis, (Erdheim–Chester disease); infectious diseases due to bacteria, mycobacteria, fungi, and rarely parasites, as well as idiopathic lesions, for example, Langerhans' cell histiocytosis. In addition, posttraumatic dysfunction of the hypothalamic–pituitary axis, prior pituitary surgery or radiotherapy, and even genetic or familial abnormalities should be taken into consideration in the differential diagnosis of hypopituitarism [13,14].

CLASSIFICATION OF PITUITARY TUMORS BASED ON IMAGING AND OPERATIVE FINDINGS Given its strategic location at the skull base, information regarding pituitary tumor size, location, extension, and invasion is necessary when one wishes to draw conclusions impacting treatment and prognosis. Classification of pituitary adenomas on the basis of size and invasiveness is determined largely by imaging studies (magnetic resonance imaging, computed tomography, conventional radiography) as well as by intraoperative findings. By convention, pituitary tumors <1 cm in their greatest diameter are considered microadenomas, whereas larger examples are termed macroadenomas. In this regard, the radiologic classification of Hardy is easily applied and clinically useful [15]. It takes into consideration not only tumor size, but extension, configuration, and invasiveness as well. Microadenomas are designated grade 0 or grade I tumors, depending on whether the sellar configuration is normal or altered in a minor way. Macroadenomas

causing diffuse sellar enlargement, focal destruction or extensive erosion of the skull base are referred to as grade II, III, and IV, respectively. Macroadenomas are further subclassified on the basis of their extrasellar extensions, whether suprasellar, parasellar, inferior, or a combination of these.

CLASSIFICATION OF PITUITARY TUMORS BASED ON ROUTINE HEMATOXYLIN&EOSIN (H&E) STAIN Once a diagnosis of pituitary adenoma has been established on the basis of clinical, laboratory, and/or imaging findings, therapeutic decision making begins. Options include surgical resection, receptor-mediated pharmacotherapy, and radiation treatment. Although some latitude exists for the initial use of medical treatment in selected endocrine-active pituitary adenomas (PRL, GH, and TSH-producing adenomas), given its rapid and consistent beneficial effects, surgery remains the initial therapy of choice in most instances. One important indication for surgery is the need of tissue for pathologic characterization. In the majority of cases the distinction of pituitary adenoma from nontumorous gland can readily be made. This is of obvious importance, but can be challenging given the small and fragmented nature of many specimens obtained transsphenoidally. After exclusion of normal compressed or hyperplastic adenohypophysial tissue the nature of the adenoma has to be assessed [16–19].

Grossly, pituitary tumors are tan-gray to purple in color and creamy in texture, contrasting with the relative firmness of the normal gland. The latter accounts for the reluctance with which normal tissue can be smeared. The histologic growth pattern of pituitary adenomas varies, ranging from diffuse to sinusoidal or papillary due to a tendency to perivascular pseudorosette formation. The recognition of these histologic details is of importance only in view of the spectrum of sellar lesions that enter into the differential diagnosis of pituitary adenoma. Given the diversity of pathologic processes that clinically and radiologically masquerade as a primary tumor, an optimal specimen devoid of artifacts is essential. We avoid frozen sections and far prefer touch or smear preparations in which the distinctive cytologic monomorphism of adenoma usually permits a ready diagnosis.

The most important routine method, the gold standard for diagnosis of pituitary adenomas, is the H&E stain. On histologic sections as well, the most important characteristics of pituitary adenoma are cellular monomorphism and lack of acinar organization. In contrast, the cells of the normal adenohypophysis are organized in a delicate acinar pattern, each acinus consisting of an admixture of different cell types surrounded by well-defined reticulin-rich network of anastomosing capillaries with fenestrated endothelium. According to their staining properties, adenohypophysial cells have been traditionally classified in three categories corresponding to acidophilic, basophilic, and chromophobic types [17,19].

Pituitary adenomas are usually well demarcated and consist of compressed nontumorous adenohypophysis (by a pseudocapsule) with condensed stroma. Unlike many benign tumors of other locations, pituitary adenomas have no "true" or fibrous capsule. Some histologically benign tumors have an indistinct border wherein clusters of adenoma cells extend into the adjacent nontumorous adenohypophysis.

The pale-staining posterior pituitary lobe is composed of nerve fibers, their expansions (Herring bodies) and terminations filled with neurosecretory material, and delicate, functionally specialized astrocytes (pituicytes).

In the H&E-stained sections minor variations in normal pituitary anatomy can be seen. None are of clinical significance. Common among these variables is so-called *basophil invasion*, which consists of migration of basophilic adenohypophysial cells of PI origin into the posterior lobe. Accumulation of such cells increases with age and appears to begin at the interface of the anterior and posterior lobes and may be impressive in extent. Occasional examples mimic pituitary adenoma. Given the origin of the anterior pituitary from stomatodeum, the finding of *salivary gland remnants*, usually on the upper surface of the posterior lobe, is not surprising. Microscopically, they resemble serous acini. Such rests may be the basis of rare salivary gland tumors of the sellar region. *Rathke's cleft remnants* are frequently encountered as glands and cleft-like spaces at the interface of the anterior and posterior lobes. Cells comprising the wall of such structures may be cuboidal, columnar, mucin-producing, or ciliated, or sometimes of adenohypophysial type. Progressive accumulation of secretions within such cysts gives rise to Rathke's cleft cysts, either sizable and symptomatic or small and incidental autopsy findings. *Intravascular hyaline bodies* are on occasion seen in the capillaries of pituitary stalk wherein they appear as eosinophilic, cylindrical, hyaline bodies resembling intravascular thrombi. *Lymphocytic foci* are seen in somewhat over 10% of normal pituitaries usually between the anterior and posterior lobes (interlobar groove). Histologically, their extent pales in comparison with the often destructive infiltrate seen in lymphocytic hypophysitis.

On the basis of cytoplasmic staining affinity using the H&E method, pituitary tumors were once classified in three categories: acidophilic, basophilic, and chromophobic adenomas. Perhaps a result of its simplicity and convenience, this approach to classification endured for decades. It is now obsolete, as much clinical and pathological overlap in functional tumor types occurs within these elementary categories. The scheme assumed that acidophilic adenomas were GH secreting and that basophilic adenomas producing ACTH-chromophobic lesions were hormonally inactive. With the emergence of new methodology, however, it became all too clear that the tinctorial characteristics of the cell cytoplasm correlates poorly with reliable cell type recognition, secretory activity, or cytogenesis. Thus, not all acidophilic tumors produce GH, nor are all GH-producing tumors acidophilic; some basophilic tumors do not cause Cushing's disease; and more than half of chromophobic tumors are endocrinologically active, variously secreting GH, PRL, ACTH, TSH, LH/FSH, and/or α-subunit.

In addition to the H&E stain, silver stain for reticulin fibers and periodic acid-Schiff (PAS) technique aid in the identification of pituitary tumors. Whereas the latter is perhaps most useful, as it shows not only positivity in ACTH adenomas and some glycoprotein hormone-producing tumors but also highlights basement membranes of the capillary network. On the other hand, silver stains show only lack of reticulin fibers in adenomas equated with lack of the acinar pattern, a classic diagnostic feature of adenomas. Silver stains are also preferred for the demonstration of pituitary hyperplasia, as its main morphological feature is the expansion of acini (Fig. 2).

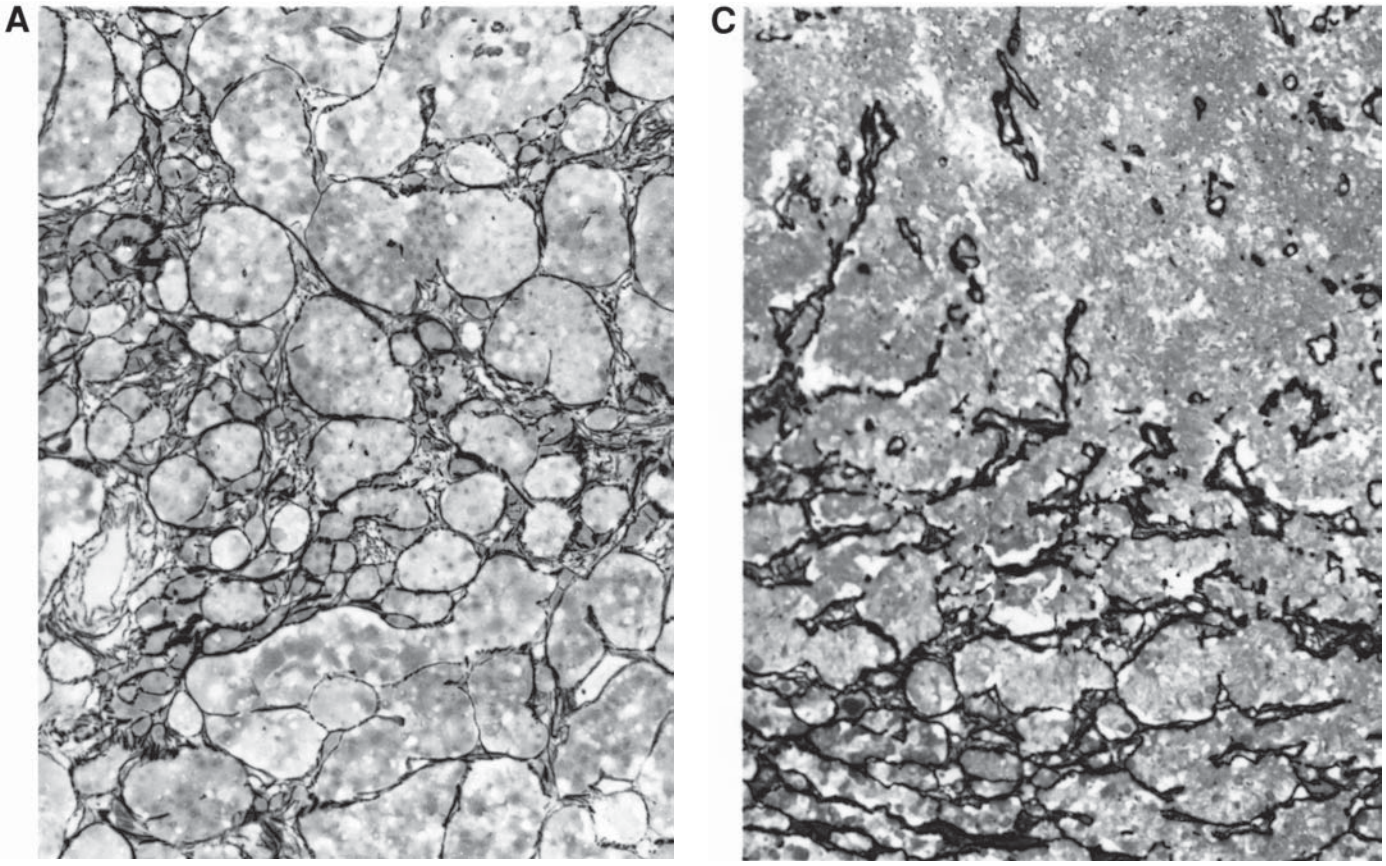

Figure 2 The Gordon–Sweet silver technique for reticulin staining is useful in distinguishing hyperplasia (**A**) from normal adenohypophyseal tissue (**B**) or from adenomas (**C**). Whereas normal adenohypophysis (**B**) displays a regular network of delicate reticulin fibers, hyperplasia (**A**) causes enlargement, distortion, and confluence of acini. In contrast, loss of acinar structure is evident in pituitary tumors (**C**). Original magnification ×100.

ADENOHYPOPHYSIAL CELL HYPERPLASIA

By definition, hyperplasia is a numerical, quantifiable increase of one or occasionally two cell types in response to physiologic demands. Attendant cytologic changes may also be seen. Only occasionally does neoplastic transformation supervene upon the hyperplastic process. Physiologic hyperplasia regularly affects the pituitary, the best example being hyperplasia of PRL cells in pregnancy and lactation. Several disease states are also accompanied by pituitary hyperplasia.

Pituitary hyperplasia is infrequent, not readily recognized, and often undiagnosed. The diagnostic difficulty is compounded by regional variation in the distribution of several pituitary cell types, inadequate or poor surgical specimens, and lack of precise diagnostic criteria for some forms of hyperplasia. From the morphologic point of view three types of pituitary hyperplasia can be distinguished [20,21].

Diffuse pituitary hyperplasia consists of a numerical increase of secretory cells without major alterations in cell morphology and acinar architecture on silver stain. When diffuse pituitary hyperplasia is marked, the acini may be slightly but rather evenly enlarged without nodularity. When not pronounced, this morphologic type may be difficult or even impossible to recognize

in fragmented specimens. Only tedious cell counts in large specimens or autopsy glands can confirm the presence of diffuse hyperplasia.

Focal pituitary hyperplasia represents a small, circumscribed accumulation of a single pituitary cell type. Such minute nodules are usually incidental findings in intact autopsy specimens. They have no apparent clinical basis and are of no significance in surgical pathology.

Nodular pituitary hyperplasia is a more advanced, widespread form of focal pituitary hyperplasia. Depending on the degree of cell proliferation, participating acini are variably enlarged and populated by an increased number of the affected cell type. If the changes are marked, focal disruption of the reticulin network and confluence of the acini take place. It is important to note that the hyperplastic mass is almost never monomorphous, other cell types being intermingled.

In general, pituitary cell hyperplasia involves cells of a single type. Only on occasion is more than one cell type affected simultaneously. The most common form of hyperplasia involves the PRL-producing cells. Not only is PRL cell hyperplasia seen in physiologic situations such as pregnancy and lactation, but it may also be associated with various pathologic processes, for example, as a component of stalk section effect, adjacent to occasional ACTH-producing adenomas, and in long-standing primary hypothyroidism where it results from the trophic effects of TRH. In contrast, GH cell hyperplasia is rare, occurring mainly as the result of an extrapituitary GH-RH-producing neuroendocrine tumor, for example, pancreatic islet cell tumor, pheochromocytoma, bronchial carcinoid, and so forth. Hyperplasia of TSH cells occurs exclusively in the context of long-standing primary hypothyroidism. LH/FSH cell hyperplasia is rare and difficult to recognize. It is well seen in patients with various forms of long-standing primary hypogonadism, for example, Klinefelter's and Turner's syndromes. ACTH cell hyperplasia does occur but its importance as a cause of Cushing's disease is still controversial. ACTH cell hyperplasia is a regular feature of untreated Addison disease and of CRH-producing extrapituitary tumors.

CLASSIFICATION OF PITUITARY TUMORS BASED ON THEIR IMMUNOHISTOCHEMICAL ASSESSMENT The development of immunohistochemistry permits the conclusive identification of the various cell types in the adenohypophysis [22]. As a result, it was pivotal in the establishment of a functional classification of pituitary adenomas and in their ultrastructural characterization. Correlation with clinical features and endocrine activity also became possible. The standard immunohistochemical battery includes the use of antibodies to GH, PRL, ACTH, TSH, FSH, LH, and the α-subunit of the glycoprotein hormones. Based on immunohistochemistry five different cell types producing six adenohypophysial hormones became recognized. Of the five known anterior lobe cell types, two, somatotrophs (GH cells) and lactotrophs (PRL cells), belong to the "acidophilic series," whereas the three other cell types, corticotrophs (ACTH cells) and other derivatives of the POMC-producing cell line, thyrotrophs (TSH cells), and gonadotrophs (FSH and/or LH cells)—belong to the "basophilic series." The anatomical regional distribution of the various cell types varies within the gland, making it difficult to quantitate cell numbers based on the examination of small tissue fragments. Somatotrophs comprise approx 50% of adenohypophysial cells and are located mainly in the "lateral wings" of the PD. Thus, somatotroph adenomas generally arise at this site. Lactotrophs represent 10–25% of adenohypophysial cells and are maximally concentrated in the posterior aspect of the lateral wings, just anterior to the neural lobe. Most lactotroph adenomas originate in this area. Corticotrophs represent 10–15% of all adenohypophysial cells, the majority of which reside within the central or "mucoid wedge." This is the usual site for functioning corticotroph adenomas. Corticotrophs in the region of Rathke's cleft and in the posterior lobe (*see* basophil invasion above) presumably give rise to nonfunctioning or "silent" corticotroph cell adenomas. Thyrotrophs, accounting for fewer than 5% of all adenohypophysial cells, occupy a small zone in the anteromedial region of the central wedge. Although thyrotroph adenomas are seldom discovered while still microadenomas, most originate at this site. Gonadotrophs are widely distributed throughout the PD, having no favored site of accumulation. As such, gonadotroph adenomas do not have a predictable site of origin.

Hormone immunohistochemistry aside, great efforts have been made to determine whether pituitary adenomas could be ascribed to a generic immunophenotype that would reliably distinguish specific adenoma types. It is now clear that the demonstration of immunoreactivity for pituitary hormones is the simplest diagnostic method of doing so, particularly in clinically nonfunctioning adenomas. For the basic diagnosis of pituitary adenoma, histology and immunohistochemistry at the light microscopic level correlate optimally with clinical, imaging, and operative findings. However, the use of transmission electron microscopy is essential to classify pituitary tumors precisely, and to determine their cytogenesis, degree of differentiation, and cellular makeup (Fig. 3).

CLASSIFICATION OF PITUITARY TUMORS BASED ON ULTRASTRUCTURE Although this approach is time consuming, expensive, and requires considerable expertise, electron microscopy provides valuable information regarding the cellular composition, cytogenesis, and secretory activity of a tumor. Using transmission electron microscopy, pituitary adenomas can be distinguished from non-neoplastic lesions and from tumors of nonadenohypophysial origin [17,19,21,23,24]. A shortcoming of ultrastructural investigation relates to small sample size, which introduces the possibility of "sampling error."

GH-PRODUCING ADENOMA

GH excess manifests in two clinically related phenotypes. The first and more common of the two is acromegaly, the result of sustained GH excess that begins or persists after puberty [1,17,19]. When GH excess manifests prior to epiphysial closure, the result is excessive linear growth or gigantism. Despite the multisystem nature of GH excess and the often dramatic physical transformation is produced, this disorder is seldom diagnosed at an early stage. Thus, pituitary adenomas underlying acromegaly or gigantism have generally progressed to the macroadenoma stage at diagnosis. Pituitary tumors associated with hypersecretion of GH are heterogeneous and can be separated into five distinct adenoma types showing differences in

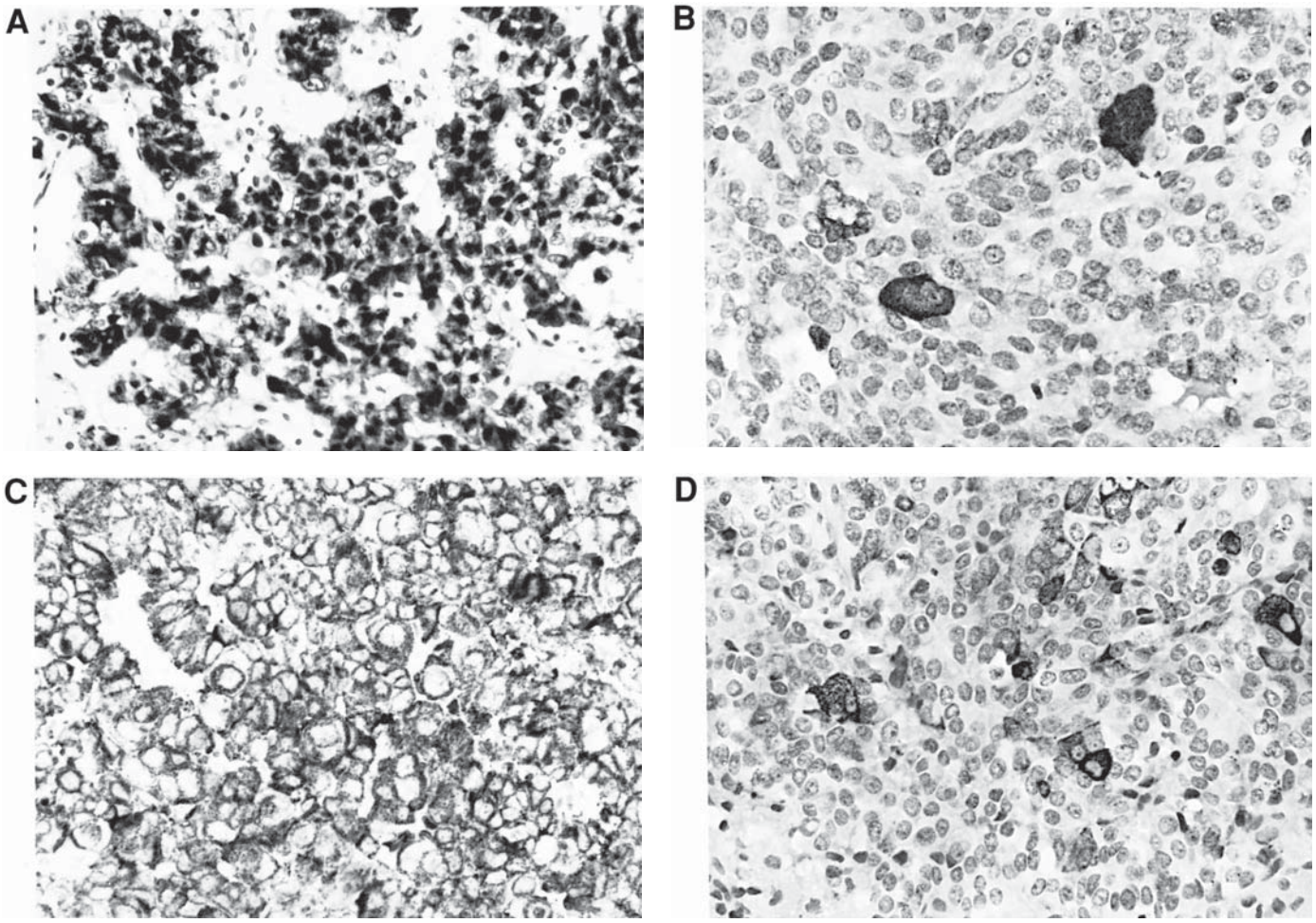

Figure 3 In some adenoma types immunostaining for specific adenophypophysial hormones may not be sufficient for conclusive diagnosis; it may even be misleading. In such cases electron microscopy is mandatory for correct diagnosis. (**A**) Immunoreactivity for PRL in a prolactin cell adenoma showing the specific Golgi immunostaining pattern. (**B**) Silent subtype 3 adenoma may exhibit immunostaining for prolactin in scattered tumor cells. However, the positivity is noted over the entire cytoplasm. (**C**) Corticotroph adenoma (*see also* Fig. 4) showing intense ACTH immunostaining in the cytoplasm of adenoma cells. (**D**) In gonadotroph adenomas of the female type (*see also* Fig. 5) a light, scattered immunostaining for ACTH may be apparent. Original magnification ×400.

incidence, immunohistochemical profile, ultrastructural morphology, and biologic behavior. Of the five types, two are monomorphous GH cell adenomas composed of either densely or sparsely granulated GH cells. The remainder are plurihormonal tumors that include mammosomatotroph adenoma, mixed GH–PRL cell adenoma, and acidophil stem cell adenoma. The latter are discussed in a separate section below.

DENSELY GRANULATED GH CELL ADENOMA These tumors comprise what has been termed the "classic acidophilic adenoma of acromegaly." It accounts for approx 8% of all pituitary adenomas, and is characterized by a relatively slow growth rate, limited invasiveness, and an overall indolent biologic course. Strong uniform cytoplasmic immunoreactivity for GH is evident in most adenoma cells. Reactivity may also be seen for PRL, α-subunit, and/or TSH. Ultrastructural analysis shows this tumor to consist of uniform, polyhedral, or elongate cells with a predominantly spherical or ovoid nuclei. The adenoma cells contain a full complement of cytoplasmic organelles including well-developed Golgi and rough endoplasmic reticulum (RER). The most prominent ultrastructural feature of this tumor is abundance of mature, GH-containing cytoplasmic secretory granules measuring 150–600 nm (mainly 400–500 nm) in diameter.

SPARSELY GRANULATED GH-CELL ADENOMA This tumor corresponds to the chromophobic variant of somatotroph adenoma. Slightly more common than the acidophilic form, it is more prevalent in women and is known to be more aggressive and rapidly growing. Immunoreactivity for GH is often limited to the Golgi zone, whereas positivity in the rest of the cytoplasm is but moderate to weak. Ultrastructural features of this tumor include scant secretory granules measuring 100–200 nm. The most distinctive feature of its cells is the presence of a so-called fibrous body, which, composed of an admixture of intermediate (cytokeratin) filaments and smooth endoplasmic reticulum (SER), is located in the Golgi region, and often indents the nucleus [25].

In GH cell adenomas treated with long-acting somatostatin analogs, mild cell shrinkage, accumulation of lysosomes, and

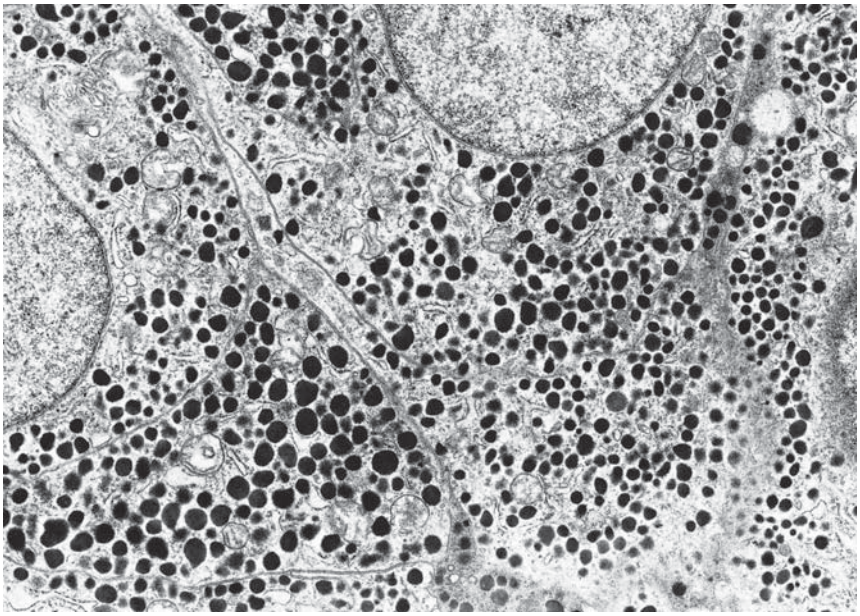

Figure 4 Corticotroph adenoma. The cytoplasm contain numerous secretory granules displaying characteristic morphology. Original magnification ×11,760.

interstitial as well as perivascular fibrosis can often be seen. These alterations are inconsistently present and are usually not marked.

PRL CELL ADENOMAS

This most frequent form of pituitary adenomas is also the most common primary tumor affecting the pituitary. Its clinical presentation relates either to the hormonal consequences of hyperprolactinemia or, particularly in postmenopausal women and in males, to neurological symptoms due to significant tumor size. The principal endocrine features of hyperprolactinemia include amenorrhea, galactorrhea, and infertility in women and decreased libido and impotence in men [17,19,26].

PRL cell adenomas are either chromophobic or amphophilic with a sizable pale Golgi zone. Distinctive psammomatous calcification is seen in a minority of tumors. Production of "endocrine amyloid" may also be encountered. Immunohistochemistry shows prolactinomas to be monohormonal tumors containing only immunoreactive PRL. Most show a characteristic paranuclear pattern of PRL immunopositivity corresponding to the conspicuous Golgi region. Only in a small minority of cases is diffuse cytoplasmic immunostaining for PRL a feature. Thus, two ultrastructural types of PRL cell adenoma are recognized.

SPARSELY GRANULATED PRL CELL ADENOMA This is the most frequent tumor type (Fig. 4). Its cells have the same striking appearance of hormonal activity as nontumorous PRL cells, abundant RER often in large concentric whorls, and prominence of the Golgi apparatus. The latter often contains pleomorphic, immature secretory granules. Secretory granules are generally sparse, measuring 120–300 nm. The ultrastructural hallmark of sparsely granulated PRL cell adenoma is the presence of granule exocytosis, the extrusion of secretory granules. These are often "misplaced exocytosis," taking place at the lateral cell surfaces, far from the vascular pole of the cell.

DENSELY GRANULATED PRL CELL ADENOMAS These are rare and may be associated with short-term dopamine agonist therapy. Otherwise, they share identical clinical, biochemical, and prognostic profiles with sparsely granulated variant. Densely granulated PRL cell adenomas contain abundant cytoplasmic secretory granules, thus their acidophilic appearance on H&E stain and diffuse cytoplasmic PRL immunopositivity. Secretory granules are spherical oval to irregular in configuration, and both larger (600 nm) and more numerous than those of the sparsely granulated variant.

The decreasing prevalence of PRL cell adenomas in surgical material is attributed to a major shift in management of these tumors from surgical toward medical therapy with dopamine agonists, such as bromocriptine or pergolide. Such medical treatment results in a striking morphologic change. In contrast to the uniform morphology of untreated tumors, PRL cell adenomas exposed to dopaminergic agonists display smaller cells in which PRL immunopositivity is scant or barely detectable. With protracted treatment, a decrease in cytoplasmic volume results in a "small cell" appearance, and marked perivascular and interstitial fibrosis. By electron microscopy, the tumor consists of small cells with markedly heterochromatic, multiply indented nuclei, and a narrow rim of cytoplasm possessing few membranous organelles, scattered lysosomes, and only few randomly distributed secretory granules. Some tumors contain a mixed population of suppressed cells and cells displaying varying degrees of endocrine activity, a feature of nonuniform involution. Cessation of treatment brings about a reversal of these changes.

ACTH CELL ADENOMAS

The majority of corticotroph adenomas are basophilic and display strong positivity with the PAS method. Immunohistochemistry demonstrates the presence of ACTH and other POMC-

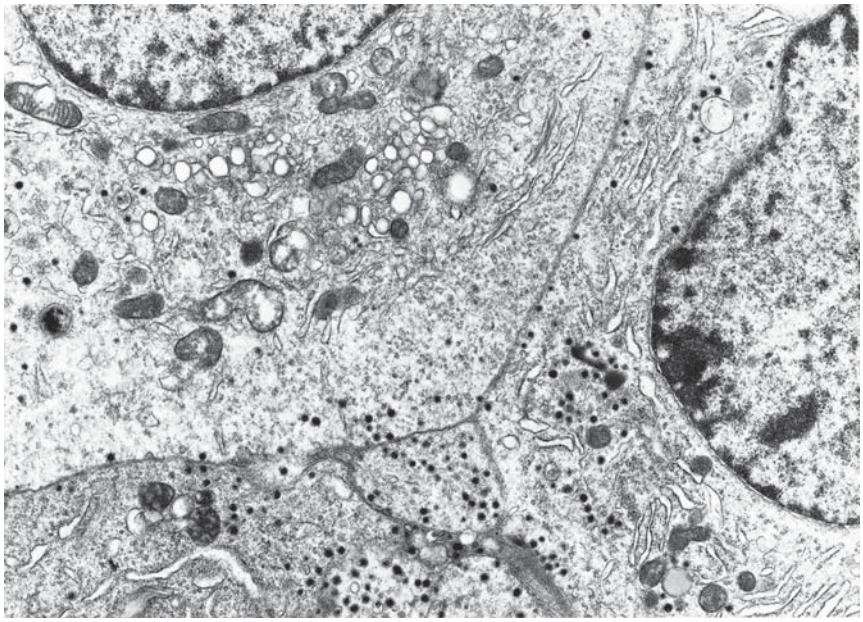

Figure 5 Gonadotroph adenomas of the female type comprise polar cells with long processes containing most of the small secretory granules. The Golgi complex shows vacuolar transformation (honeycomb Golgi). Original magnification ×13,640.

related peptides in the cytoplasm of adenoma cells [17,19,27]. Corticotroph adenomas are most often monomorphous and monohormonal. Rarely, however, they exhibit immunopositivity for α-subunit, LH, or PRL. Typical corticotroph adenomas are associated with signs and symptoms of corticosteroid excess (Cushing's disease), that is, moonlike facies, acne, hirsutism, truncal obesity, abdominal striae, easy bruising, mood changes, hypertension, osteoporosis, insulin resistance, diabetes mellitus, and muscle weakness. Such tumors exhibit a marked female preponderance. Only about half of the adenomas in Cushing's disease are detectable by imaging procedures; the remainder are very small. Macroadenomas are uncommon in Cushing's disease and are usually invasive and difficult to cure. The same is true of a subset of Cushing's adenomas that were treated by adrenalectomy (Nelson's syndrome). Such tumors may have been radiographically undetectable at presentation or aggressive sizable adenomas from the start. In any event, unlike the tumors of Cushing's disease, those of Nelson's syndrome are usually invasive macroadenomas associated with hyperpigmentation (melanocyte-stimulating hormone effect), visual field defects, and headaches. A large proportion of pituitary carcinomas have their origin in Nelson's syndrome.

Although the histologic and immunohistochemical appearance of tumors associated with Nelson's syndrome is similar to the previously described adenomas of Cushing's disease, they do show slightly different ultrastructural features. In corticotroph adenomas associated with Cushing's disease the adenoma cells are elongated or angular with ovoid nuclei showing occasionally indentations. The cytoplasm is abundant and contains prominent RER, free ribosomes, and polysomes, as well as a conspicuous Golgi complex. Secretory granules measuring 150–450 nm are numerous and exhibit highly characteristic morphology, being spherical, teardrop, or heart shaped and showing variable electron density (Fig. 5). The other characteristic ultrastructural marker is bundles of keratin immunoreactive intermediate filaments disposed around the nucleus [28–30]. Excessive accumulation of these filaments, a phenomenon referred to as Crooke's hyalinization, usually occurs in surrounding nontumorous corticotroph cells. On occasion, the adenoma cells may show Crooke's change, filaments occupying large areas of the cytoplasm, displacing organelles and secretory granules to the cell periphery. Tumors composed of Crooke cells are termed Crooke's cell adenomas. They are typically Cushing's disease associated, often invasive, and recur more frequently than other ACTH cell adenomas. In Nelson's syndrome the electron microscopic features of the tumor cells are similar to those seen in Cushing's disease, but with one important exception—that high levels of cortisol are not a feature of Nelson's syndrome, and because Crooke's hyaline change is the negative feedback effect of elevated glucocorticoid levels, intermediate filaments are lacking in Nelson's adenomas.

TSH CELL ADENOMA

This tumor is rare, representing only about 1% of all pituitary adenomas [31–33]. Clinically, most TSH cell adenomas present with the signs and symptoms of hyperthyroidism. The thyroid gland is diffusely enlarged. The diagnostic hallmark is the presence of an inappropriately high TSH level in the presence of elevated peripheral thyroid hormone concentrations. A minority of tumors occur in the setting of hypothyroidism. From the histopathologic standpoint, the diagnosis of thyrotroph adenomas is often difficult due to their variable morphology. Generally, they are composed of chromophobic, angular-shaped cells disposed in a sinusoidal or diffuse pattern. Interstitial and perivascular fibrosis may be conspicuous in some cases. By immunohistochemistry, the cells are positive for TSH and often α-subunit. A minority of thyrotroph adenomas also show variable

reactivity for GH and/or PRL. At the ultrastructural level, the TSH cells are spindle-shaped and possess long cytoplasmic processes as well as spherical to ovoid nuclei, often with prominent nucleoli. The cytoplasm contains moderately developed organelles and small (50–200 nm) secretory granules peripherally situated beneath the cell membrane.

FSH/LH CELL ADENOMA

Gonadotroph adenomas may be associated with increased serum levels of FSH, LH, and/or α-subunit, but the majority of patients have gonadotropin levels within normal limits for their age. Most tumors occur in middle and older age, men being more often affected. Even if a tumor is hormonally active, gonadotropin excess does not result in clinical hyperfunction. Thus, patients with this adenoma typically present with hypogonadism and symptoms of mass effect, mainly visual disturbance and hypopituitarism [17,19,34,35].

Histologically, gonadotroph adenomas are chromophobic tumors featuring pseudorosettes and papillae. Microcysts may also be evident. PAS stains may highlight the presence of secretory granules beneath the cell membrane. Differences in the pattern of immunostaining may be seen in men and women. Adenomas of men are more likely to demonstrate immunoreactivity for FSH and/or LH, staining being variable and often unevenly distributed. In contrast, tumors in women are often poorly immunoreactive, some showing scant if any staining for gonadotropins. Among pituitary adenomas, gonadotropic tumors are the only ones exhibiting sex-linked differences in ultrastructural appearance. The so-called "male type" possesses slightly dilated RER, a prominent Golgi complex with sparse, small (200 nm) secretory granules, and, in 50% of cases, varying degrees of oncocytic changes. In contrast, gonadotroph adenomas of the "female type" feature a unique morphological marker, the so-called "honeycomb Golgi complex" in which the sacculi transform into clusters of spheres containing a low-density proteinaceous substance.

SILENT ADENOMAS

The term "silent adenoma" has been applied to three clinically nonfunctioning tumors, each morphologically distinct from null cell adenomas. Unlike the latter, silent adenomas consist of cells often showing well-defined immunoreactivity for hormones, most frequently ACTH. In contrast, null cell adenomas are immunonegative or contain only few cells immunopositive for FSH/LH and/or α-subunit. Two of the three silent adenomas show morphologic resemblance to the corticotroph adenomas of Cushing's disease, whereas null cell adenomas reflect endocrine differentiation, but show no markers of any specific pituitary cell type. At present, aside from immunohistochemistry, electron microscopy is required for the conclusive identification of the silent adenomas, particularly those of subtype 3 [17,19,36,37].

SILENT "CORTICOTROPH" ADENOMA SUBTYPE 1 Morphologically these tumors are indistinguishable from the adenomas of Cushing's disease. The amphophilic, PAS-positive tumor cells are immunoreactive for ACTH and other POMC-related peptides. Ultrastructurally, there are similarly no differences between the two lesions. However, an unusual and unexplained characteristic of silent corticotroph adenoma subtype 1 is the frequent occurrence of hemorrhage and infarction. Recent findings point to an origin of this tumor from PI-derived POMC cells, the function of which is still unknown.

SILENT "CORTICOTROPH" ADENOMA SUBTYPE 2 This primarily affects men. Histologically, most are chromophobic and, in contrast to subtype 1 "corticotroph" adenomas, show only mild, patchy PAS-staining and ACTH immunoreactivity. Positivity for β-endorphin is often stronger. Ultrastructurally, the tumor is less obviously corticotropic. Its cells are polyhedral without polarity, contain secretory granules smaller (200–350 nm) than those of ACTH-secreting and silent subtype-1 adenomas, and lack intermediate filaments. On the other hand, the secretory granules are similar to those of Cushing's and silent subtype 1 adenomas.

SILENT ADENOMA SUBTYPE 3 This intriguing tumor type is a nosologic enigma. Its clinical presentation and morphologic features have been well characterized; yet the issue of histogenesis remains to be settled. It was originally thought to be related to the two previously discussed silent adenomas based on variable but usually scant immunoreactivity for ACTH and other POMC-related peptides in some examples. Most tumors are immunonegative for ACTH. More often, one sees scattered immunoreactivity for GH, PRL, and α-subunit. Lastly, some tumors are entirely immunonegative. The ultrastructure of silent adenoma subtype 3 is complex. It is composed of large, polar cells, the cytoplasm of which contains RER, often copious amounts of SER, and a very well developed Golgi apparatus. Secretory granules vary in number. Measuring about 200 nm, they often collect at one pole of the cytoplasm, as is the case with well-differentiated glycoprotein hormone-producing cells. Based on these ultrastructural features, the tumors seem to be actively secreting, but what is being produced remains to be determined. In view of their variable, confusing immunophenotype, the diagnosis requires ultrastructural confirmation.

NULL CELL ADENOMAS

Null cell adenomas are mainly in adults, particularly the oncocytic variant. The term "null" signifies the lack or paucity of morphological, especially ultrastructural, markers that would indicate either a cell of origin or a direction of differentiation [1,17,19]. Histologically, these tumors vary from chromophobic to eosinophilic and granular (oncocytic) and exhibit either a diffuse pattern or pseudorosette formation. Immunostains are often negative or show only scattered positivity for one or more hormones, usually combinations of FSH, LH, or α-subunit. On occasion, scattered cells even show immunoreactivity for GH, PRL, or ACTH. Although null cell adenomas may be immunonegative for hormones and lack function, endocrine differentiation is evident as reactivity for neuron-specific enolase, chromogranin, and/or synaptophysin. At the ultrastructural level, null cell adenomas vary. Chromophobic tumors are composed of cells with small quantities of cytoplasm containing poorly developed RER and Golgi, as well as scant, small (100–250 nm) secretory granules. The cells of the oncocytic variant are larger. Their sole ultrastructural characteristic is the excessive mitochondrial accumulation. Despite marked mitochondrial abundance, the same RER and Golgi as well as secre-

tory granules are always evident. In those tumors containing somewhat more differentiated cells, these usually show features of glycoprotein hormone–producing cells. This is not surprising, because from the histologic, immunohistochemical, and ultrastructural aspects, an apparent overlap exists between null cell adenomas—oncocytomas and gonadotroph adenoma, being difficult to draw the line between the two entities in many cases.

THE CONTRIBUTION OF MOLECULAR AND GENETIC TECHNIQUES TO THE STUDY OF PITUITARY TUMORS

Several novel techniques have recently been introduced to analyze the molecular and genetic aspects of pituitary tumors [38–41]. These have advanced our understanding of molecular pathogenesis of these lesions. The development of pituitary adenomas appears to be a multistep, multicausal process involving tumor initiation followed by tumor promotion. Only the most relevant findings are reviewed in the following paragraphs. These aspects of pituitary development and tumors are covered in Chapters 4 and 5.

CLONAL ORIGIN OF PITUITARY TUMORS

A fundamental and still controversial issue related to pituitary tumorigenesis is the question of whether neoplastic transformation of adenohypophysial cells is due to hypothalamic dysfunction or simply the result of an acquired mutation of a single cell. Using the allelic X-chromosome inactivation analysis, several laboratories have confirmed the monoclonal composition of virtually all pituitary adenomas. Thus, pituitary adenomas are considered monoclonal expansions of a single somatically mutated and transformed cell [42–44].

HYPOTHALAMIC FACTORS AND PITUITARY TUMORS

Despite demonstrated clonality of most if not all pituitary tumors, a contribution of hypothalamic hormones to pituitary tumorigenesis has been considered [45]. For good reason there is renewed interest in integrating their role in the current multistep monoclonal model. For example, it has been demonstrated that abnormal activity of hypothalamic hypophysiotrophic hormones, in either the form of excess stimulation or deficient inhibition, may contribute to the genesis and/or progression of pituitary tumors. It has also been shown that somatotroph hyperplasia of long-standing duration can undergo adenomatous transformation. High-level ectopic GH-RH-production in patients with GH-RH-producing extrapituitary tumors also results in somatotroph hyperplasia followed in some cases by adenoma formation [46,47]. Animal models also provide support for the notion. For example, rats transgenic for GH-RH develop somatotroph hyperplasia and subsequently pituitary adenoma. It was also shown that dopamine receptor (D_2) knockout rodents develop PRL-producing pituitary adenomas [48,49].

ENDOCRINE FACTORS

Both experimental studies and clinical investigations have provided evidence that endocrine abnormalities may predispose, promote, or even induce the development of pituitary adenomas [1,3]. For example, thyrotroph adenomas are known to develop in patients with long-standing primary hypothyroidism as are corticotroph adenomas in untreated Addison's disease. It is also known that protracted estrogen stimulation contributes to transformation and/or neoplastic progression of PRL cell adenomas in the rodent and human pituitaries.

GENOMIC ALTERATIONS IN PITUITARY ADENOMAS

Since it became clear that somatic mutation(s) in a single adenohypophysial cells is the event requisite to pituitary tumorigenesis, vigorous attempts have been made to identify and characterize the responsible mutations [50–52]. Activating mutations of two oncogenes, *GSPT1* and H-*ras*, have been found in human pituitary adenomas. In addition, H-*ras* and c-*myc* oncogenes, as well as mutations of *p53*, *nm23*, and *Rb* genes, have been identified disproportionately more often in aggressive tumors. For example, mutation of the *Rb* gene has been seen in pituitary carcinomas. These observations provide evidence that amplification of oncogenes (H-*ras* and c-*myc*) and inactivation of tumor suppressor genes (*p53*, *nm 23*, and *Rb*) may play a role in initiation and/or tumor progression. The recent application of microarray technology has shown large number of genes in pituitary tumors to be abnormal.

PLURIHORMONALITY

The development of light microscopic immunohistochemistry and its ancillary techniques, such as double immunostaining and immunoelectron microscopy, challenged and negated the long-accepted "one cell–one hormone" theory, as they showed plurihormonality to be a common occurrence in both normal and neoplastic pituitary cells (Fig. 6) [53,54]. Although the presence of more than one hormone in the same cell was initially hard to explain, modern studies showed that precursor cells can differentiate toward the spectrum of cell types that populate the adult adenohypophysis. Current evidence suggests that corticotrophs arise as a lineage distinct from that of the other pituitary cell types. The cells belonging to other lines, for example, somatotrophs, lactotrophs, thyrotrophs, and gonadotrophs, appear to be related in that they utilize common transcription factors. This is especially true for somatotrophs and lactotrophs, because, in contrast to other cell types that function independently, lactotrophs have a strong dependence on somatotrophs. Several different transcription factors regulating the transformation of primordial pituitary cells to mature secretory cells have been identified. These include *Rpx/Hes1, Pitx1, Ptx2, Lhx3/LIM3/P-lim, Prop-1,* and *Pit-1/GH factor 1* [55,56].

It is not clear whether plurihormonal cells occur more frequently in adenomatous or in normal, nontumorous pituitaries [57]. Under physiological conditions the presence of plurihormonal cells can be related to the phenomenon of "transdifferentiation," which involves reversible transformation of one cell type to another. In neoplasms, mutation or gene deletion may lead to the development of new immunohistochemical or ultrastructural phenotypes, thus accounting for cell heterogeneity [58–60].

Plurihormonal pituitary adenomas may be monomorphous, that is, composed of a distinct morphologic cell type, which nonetheless secretes more than a single hormone. Yet other tumors consists of two or more morphologically different cell types. For example, several plurihormonal adenomas associated

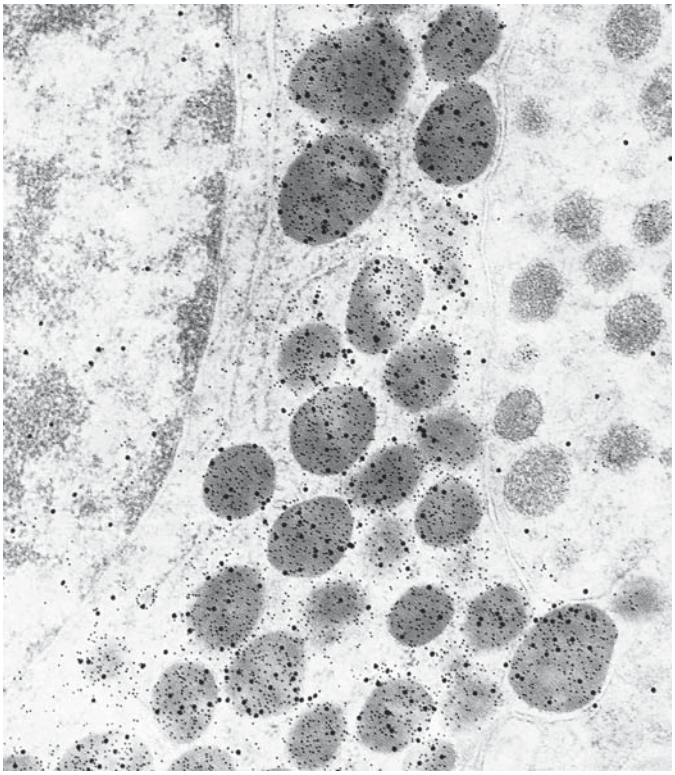

Figure 6 Double-immunogold method demonstrates colocalization of GH (imunolabeling with 10-nm gold particles) and PRL (immunolabeling with 20-nm gold particles) in the same secretory granules of mammosomatotroph in a bihormonal tumor. Original magnification ×31,160.

with acromegaly produce GH and one or more glycoprotein hormones, primarily α-subunit [61,62]. These patients have acromegaly elevated serum GH and insulin-like growth factor (IGF)-1 serum levels. Immunohistochemistry demonstrates cells producing GH and α-subunit, less often TSH, FSH, and/or LH. By electron microscopy, the appearance of tumors is chiefly monomorphous, similar to that of densely granulated somatotroph adenomas [1,17,19].

MIXED SOMATOTROPH-LACTOTROPH ADENOMAS This tumor is most commonly composed of densely granulated somatotrophs and sparsely granulated lactotrophs. On hematoxylin and eosin stained sections, it consists of acidophilic cells interspersed with chromophobic cells. By immunohistochemistry GH and PRL are demonstrated in different cell populations. Electron microscopy documents the bimorphous nature of the tumors.

ACIDOPHIL STEM CELL ADENOMA These rare, hyperprolactinemia-associated tumors tend to grow rapidly in young individuals. They are chromophobic or slightly acidophilic, immunohistochemically reactive for PRL and to a lesser extent GH, but monomorphous. In some cases, GH immunoreactivity may not be apparent. Ultrastructurally, acidophil stem cell adenomas are monomorphous but demonstrate both lactotroph and somatotroph markers, that is, granule extrusions and fibrous bodies. The tumors may be oncocytic, even in young patients, and display a unique and diagnostic form of giant mitochondria. The sparse, randomly distributed secretory granules are small (50–200 nm) [63].

MAMMOSOMATOTROPH ADENOMA Morphologically similar to densely granulated somatotroph adenomas, the tumor is monomorphous in cellular makeup and strongly acidophilic. Immunohistochemistry shows reactivity for both GH and PRL within the same cells. Staining for PRL is variable and many tumors also contain α-subunit. The diagnosis is confirmed by electron microscopy.

CELL PROLIFERATION MARKERS

Several cell proliferation markers including proliferative cell nuclear antigen (PCNA), MIB-1 (Ki-67), p-27, cyclins, topoisomerase II-α, AGNOR (argyrophilic nuclear organization region), and BrdUrd (bromodeoxyuridine), can be used to document kinetic abnormalities that play a role in tumor progression [64–66]. As detected by the MIB-1 antibody, Ki-67 expression is a useful marker of proliferative activity, invasiveness, and prognosis in a variety of tumor systems. Although many pituitary tumors show a slow rate of growth, others enlarge more rapidly and invade neighboring tissue. Only rare examples give rise to distant cerebrospinal and/or systemic metastases (pituitary carcinomas). The prognostic value of cell proliferation markers in pituitary tumors has been confirmed in several studies showing a correlation between high labeling indices and aggressive behavior. Particularly high MIB-1 and PCNA labeling is seen in metastases (pituitary carcinoma) as well as in their respective primary tumors (Fig. 7). Measurements of microvessel density show increased angiogenesis in various types of malignant tumors. Although microvessel density is lower in pituitary adenomas than in the nontumorous gland, pituitary carcinomas have increased microvessel density.

PITUITARY CARCINOMAS Pituitary carcinomas are very rare and are defined as primary neoplasms of the adenohypophysis that undergo craniospinal and/or systemic spread [5–8]. Brain invasion, a feature evident only at autopsy, is also an indicator of malignancy. The pathogenesis of pituitary carcinoma is controversial. For example, it is unclear whether carcinomas develop from adenomas or arise *de novo*. From the endocrinologic standpoint, pituitary carcinomas are more often hormone-secreting than nonfunctioning tumors. Among functioning carcinomas, the most common types are PRL- or ACTH-producing. GH-, TSH-, and FSH/LH-producing tumors are very rare. Metastatic involvement of the central nervous system is more often craniospinal leptomeningeal than parenchymal. Favored sites of systemic spread include liver, lung, bone, and lymph nodes.

Morphologically the histopathology of pituitary carcinomas varies. In some cases, the histology is indistinguishable from that of benign adenomas. Most, however, show increased numbers of mitotic figures, nuclear atypia (hyperchromasia, pleomorphism nucleolar prominence), and necrosis (Fig. 8). Cellular atypia is usually more conspicuous in the metastases than in the primary tumors. Immunohistochemistry shows the same degree of reactivity for hormones as in adenomas. In most cases, significantly increased MIB-1 labeling and microvessel density, as well as increased *P53* protooncogene expression, are noted. As noted earlier, *ras* mutations can be seen in PRL cell carcinomas.

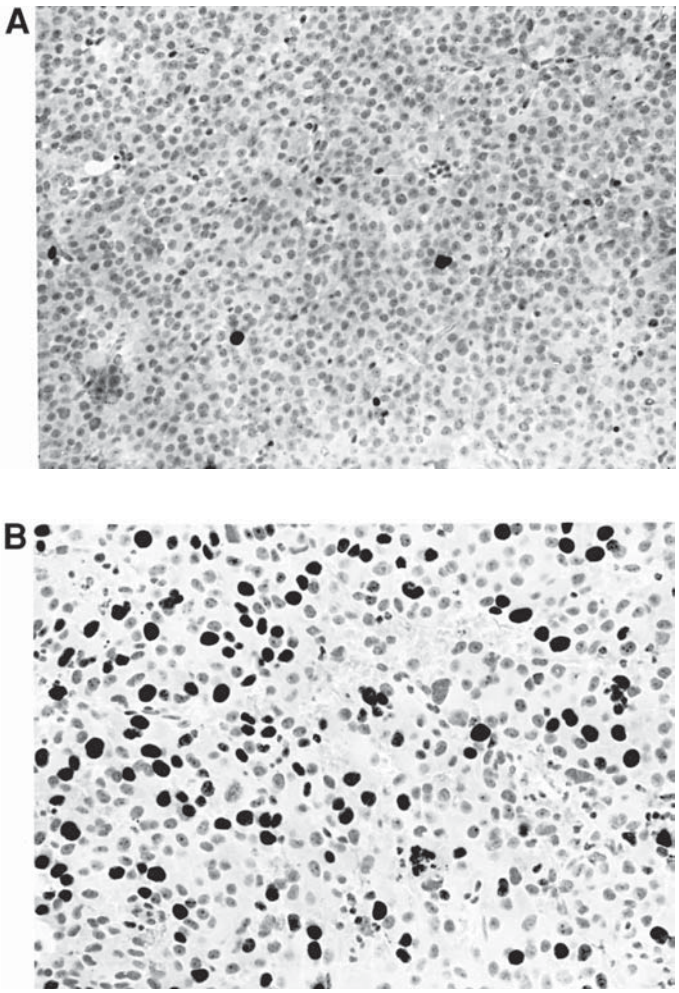

Figure 7 Although pituitary tumors generally exhibit a slow growth rate previous studies have shown a relationship between the expression of cell proliferation markers and aggressive tumor behavior. (**A**) Pituitary tumor showing a low (<1%) MIB-1 labeling index. (**B**) Pituitary tumor showing high (>7%) MIB-1 labeling index. Original magnification ×250.

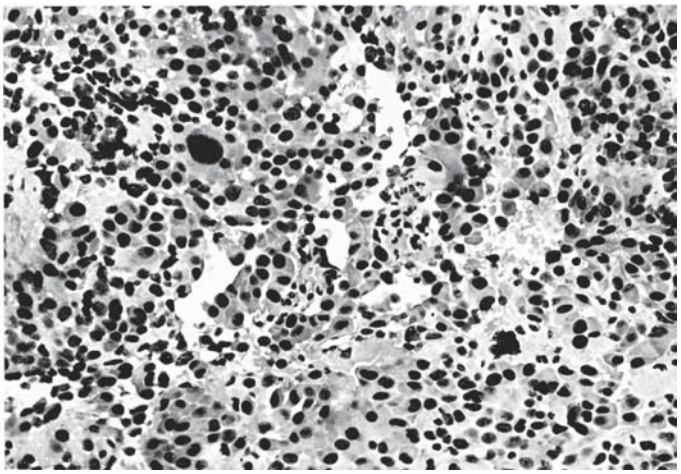

Figure 8 Pituitary carcinoma showing nuclear and cellular atypia. Original magnification ×250.

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4 Recent Developments in Molecular Embryogenesis and Molecular Biology of the Pituitary

ROBERT YOSHIYUKI OSAMURA, MD, PHD, AND NOBORU EGASHIRA, MD

INTRODUCTION

The embryogenesis and functional differentiation of the pituitary glands have been studied for more than a half century. Recent development in molecular technologies and the cloning of various of transcription factor genes have stimulated rapid progress in the clarification of commitment of pituitary cells in hormone production. The pituitary gland has been known to be derived from the oral epithelium as a primordium that gives rise to an infolding termed Rathke's pouch. Rathke's pouch is composed of the anterior limb and the posterior limb. The former develops to produce various hormones—growth hormone (GH), prolactin releasing hormone (PRL), thyroid-stimulating hormone (TSH), adrenocorticotropic hormone (ACTH), and follicle-stimulating hormone (FSH)/luteinizing hormone (LH). It is of interest that the posterior limb eventually produces only ACTH and melanocyte-stimulating hormone (MSH). This chapter reviews the development of the pituitary glands and the role of transcription factors and cofactors.

EARLY DEVELOPMENT OF THE PITUITARY GLAND

It has been demonstrated that the anterior limb of Rathke's pouch differentiates in a particular chronological order and forms the anterior lobe. It is well known that α-SU is the first hormone appearing in the proliferating anterior limb and is followed by proopiomelanocortin (POMC), GH, PRL, TSH, and FSH/LH. Among these, TSH, FSH, and LH are the glycoproteins, which are composed of common SU and morophofunctionally specific β-SU. On the other hand, the posterior limb of Rathke's pouch develops into a "bandlike" structure with predominant differentiation into α-SU. POMC (ACTH) in the anterior lobe appears earlier than that in the intermediate lobe.

In the human pituitary gland, similarly, the anterior limb develops into various hormone-producing cells. It is of particular interest that the posterior limb forms an intermediate lobe-like structure only during fetal life. In the adult pituitary, these posterior-limb-derived cells remain as "invading anterior cells" in the posterior lobe [1,2]. As in the intermediate lobe in rodents, these "invading anterior cells" differentiate predominantly to POMC (ACTH). However, immunoreactive α-SU positivity is rather rare.

The other unique event in the developing pituitary gland is the movement of POMC- differentiated cells from the periphery to the center in the anterior pituitary glands. In the adult pituitary gland, the following types of the hormone-producing cells show an intimate relationship, that is, ACTH with GH, FSH/LH with PRL (especially in females). The POMC cells show cytoplasmic processes directed to the capillaries and cytoplasmic attachment to the GH cells. Another unique finding in the human pituitary gland is that GH-producing cells are also immunohistochemically positive for α-SU and PRL.

Folliculostellate (FS) cells are unique cells in the anterior pituitary marked by the presence of S100 protein, and are easily detected by immunohistochemistry. FS cells have a particular shape determined by a few elongated cytoplasmic processes that engulf the other hormone-producing cells. Cytokine production has been recently reported [3].

DEVELOPMENT OF THE HYPOTHALAMO–PITUITARY AXIS

It has been well known that the functions of the anterior pituitary cells are under the regulation of hypothalamic factors as follows: growth hormone releasing hormone (GHRH) → GH cells, dopamine, PrP → PRL cells, thyrotropic hormone (TRH) → TSH cells, corticotrophin-releasing hormone (CRH) → POMC cells, gonadotropin releasing hormone (GnRH) → FSH/LH cells. These anterior pituitary cells possess receptors (R) for these factors from the hypothalamus, that is, GH cells—GHRH-R; PRL cells—dopamine receptor; PrPR, TSH cells—TRH-R; POMC—CRH-R; FSH/LH cells—GnRH-R. PRL cells also are well known to possess estrogen receptors (ERs) that bind to DNA. In rodents, the developmental relationship between hypothalamic factors and pituitary hormones has been studied

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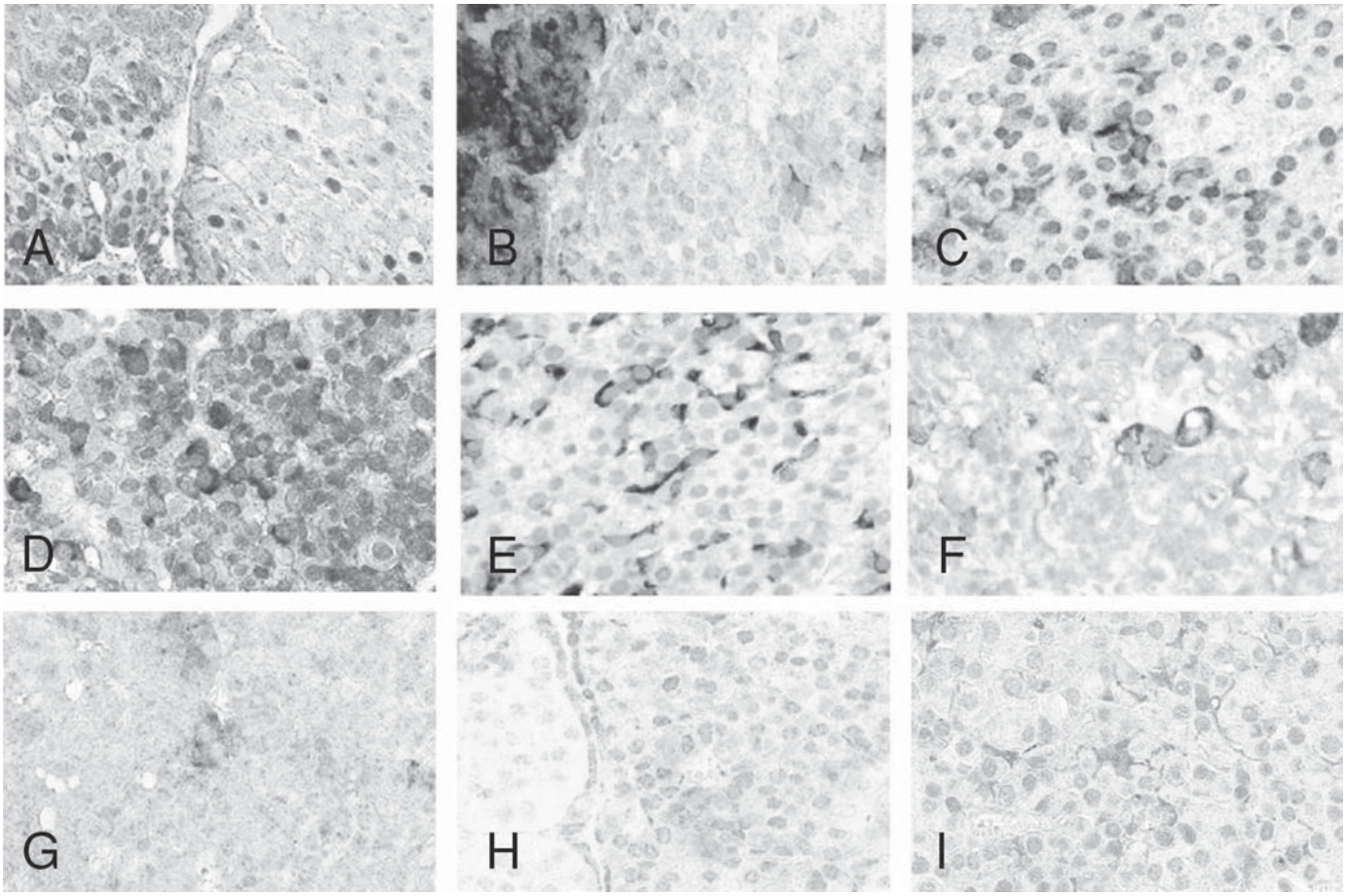


Figure 1 Immunohistochemical co-localization of *Ptx1* (brown) and pituitary hormones or S-100 protein in rat pituitary (blue). *Ptx1* was detected in the nuclei of intermediate lobe (IL) and anterior lobe (AL) (**A**). *Ptx1* was localized in the nuclei of POMC-secreting cells (**B**), ACTH-secreting cells (**C**), GH-secreting cells (**D**), PRL-secreting cells (**E**), TSH-secreting cells (**F**), αSU-secreting cells (**G**), FSH-secreting cells (**H**), and S-100-positive cells (I). (Color illustration appears in insert following p. 148.)

by immunohistochemistry (IHC) and *in situ* hybridization (ISH). It is of functional and morphologic interests that hypothalamic factors such as GHRH and CRH appear before the corresponding hormones GH and POMC, respectively. It has been shown recently that some receptors, for example, GHRH-R and CRH-R, appear before hormone production—GH and POMC. The reason for this reciprocal appearance remains to be further investigated.

DEVELOPMENT OF PITUITARY FUNCTION AND TRANSCRIPTION FACTORS

It is well established that the functions of the anterior pituitary cells are under the control of transcription factors and their cofactors such as the corresponding receptors on the cell membrane and in the nuclei. Transcription factors are divided into two groups: (1) transcription factors in early pituitary development and (2) transcription factors in later functional differentiation. The study of transcription factors first was stimulated by cloning pituitary-specific transcription factor-1 (*Pit-1/GHF-1*) and has resulted in the discovery of many subsequently cloned transcription factors, both in the early development and in the later functional stages.

TRANSCRIPTION FACTORS IN EARLY PITUITARY DEVELOPMENT *Rpx-1* is the factor that regulates the formation of Rathke's pouch and *Lhx-3* (*p-Lim, Lim3*) maintains the formation of the pouch and basic cellular structure of the pituitary glands.

Pituitary homeobox 1 (*Ptx1/Pitx1*) was first reported as a factor needed for differentiation into POMC (Fig. 1), but is currently considered to be another transcription factor that appears in early development and cooperates with the following more functionally oriented transcription factors: *Pit-1* and *NeuroD1*. *Ptx1* and pituitary homeobox 2 (*Ptx2/Pitx2*) are expressed in the primordial Rathke's pouch in the oral epithelium and throughout the development of the pituitary (Fig. 2) [4–7]. In the *Ptx1* null mutant mice, it has been observed that the development of the anterior pituitary gland is deficient and the anterior pituitary reduced expression of TSH-β α-subunit, LH-β α-subunit, FSH-β α-subunit, and α-SU but the expressions of GH and POMC are unchanged [5].

Furthermore, *Ptx2* expression overlaps with *Ptx1* in the early developing pituitary. In the pituitary of the *Ptx2* null mutant, invagination occurs normally but subsequent development is reduced [6,7]. *Rpx* is not expressed in the pituitary, but *Lhx3* is

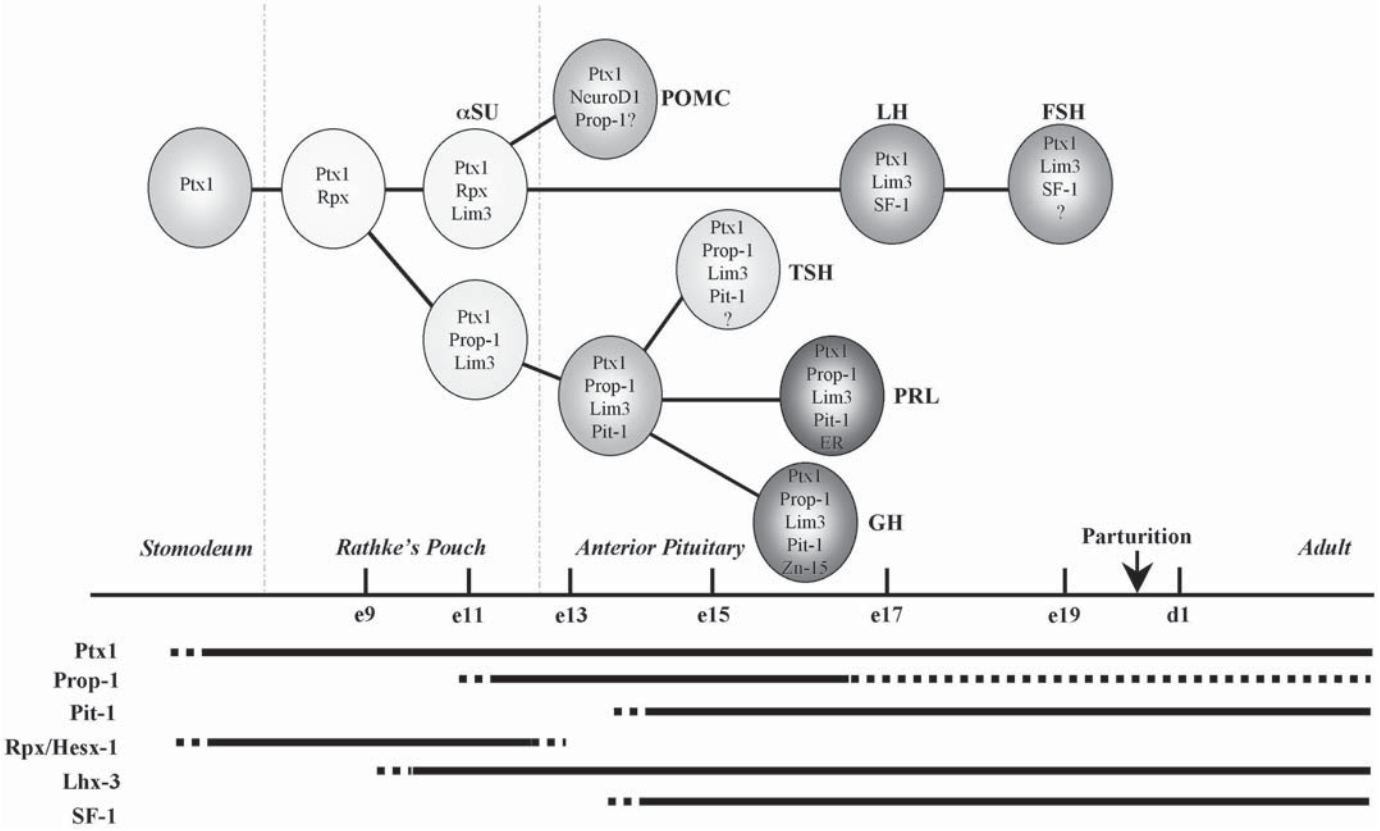


Figure 2 Cell differentiation regulated by the expression of transcription factors in the mouse anterior pituitary during embryogenesis.

expressed. The phenotype is similar to that *Lhx3*$^{+/-}$ and *Lhx4*$^{-/-}$, and the relative roles of *Ptx1* and *Ptx2* may be similar to those of Lhx3 and Lhx4 [6,7].

TRANSCRIPTION FACTORS IN LATER FUNCTIONAL DIFFERENTIATION *Pit-1* is a transcription factor that regulates the functional differentiation of the anterior pituitary cells to GH, PRL, and TSH. *Pit-1* has three exons that code proteins of a 291-amino-acid sequence and molecular mass of 33 kDa. *Pit*-1 has two isoforms with insertion of 26 amino acids (*Pit-1*β) and 14 amino acids (*Pit-1T*) (Fig. 3). The upstream promoter region of the *GH* gene has two *Pit-1* biding sites. The *PRL* and *TSH* genes contain eight and five *Pit-1* binding sites in the promoter regions respectively (Fig. 4).

NeuroD1 is a family of basic helix–loop–helix (bHLH) transcription factors and plays a role in the functional differentiation for POMC (ACTH) production. *NeuroD1* is well documented to function with *Ptx-1*, the binding site of which is located near that of *NeuroD1*. *GATA-2* is a recently cloned transcription factor that plays a role together with *Pit-1* in the differentiation to TSH (Fig. 5) [8]. Steroidogenic factor-1 (*SF-1*) is a key factor for the differentiation of LH. *DAX-1* has been regarded as a factor for LH, but a recent study has disclosed its presence in all types of pituitary cells (Fig. 2).

COFACTORS THAT WORK SYNERGISTICALLY WITH TRANSCRIPTION FACTORS The cofactors may be classified as (a) those on the cell membrane, such as receptors for hypothalamic hormones, and (b) those within nuclei, such as nuclear receptors, for example, estrogen receptors (Fig. 6A).

Cell Membrane Receptors The receptors for hypothalamic hormones are seven transmembrane protein forms and include GHRH-R and GnRH-R. *Pit-1* and GHRH-R function synergistically for the production of GH in the pituitary cells. GHRH-R has a *Pit-1* binding site in the upstream promoter region (Fig. 6B). GnRH-R stimulates LH cells with GHRH binding and results in the activation of binding sites for *SF-1* in the upstream promoter region (Fig. 7). GnRH-R may play a role in FSH cells, but detailed mechanisms still remain to be clarified further.

Nuclear Receptors The estrogen receptor (ER) is a good example of this category of receptor and functions with *Pit-1* for the production of PRL (Fig. 1D). The dopamine receptor is another factor that negatively regulates PRL production by binding to the promoter region of the *PRL* gene. The retinoic acid receptor (RAR) is a DNA binding protein for the production of GH by a synergistic function with *Pit-1*. The retinoid X receptor (RXR) is a factor that has been clarified to play a role in the functional differentiation of TSH with *Pit-1* binding (Fig. 1E,F,6A).

LOSS OF FUNCTION IN TRANSCRIPTION FACTORS. MECHANISMS AND DISORDERS

Dwarfs (*dw*) of various types include *Pit-1*, *Prop-1*, and *Pit-1* binding sites of GHRH-R. The discovery of *Pit-1* was led by analysis of the Snell dwarf mouse (*dw*), which is associated with dwarfism. The locus of the *Pit-1* gene is located on mouse chromosome 16, which is consistent with the *dw* mutation [9]. The Jackson dwarf mouse (*dw*^{J}) is associated with dwarfism as in the Snell dwarf mouse.

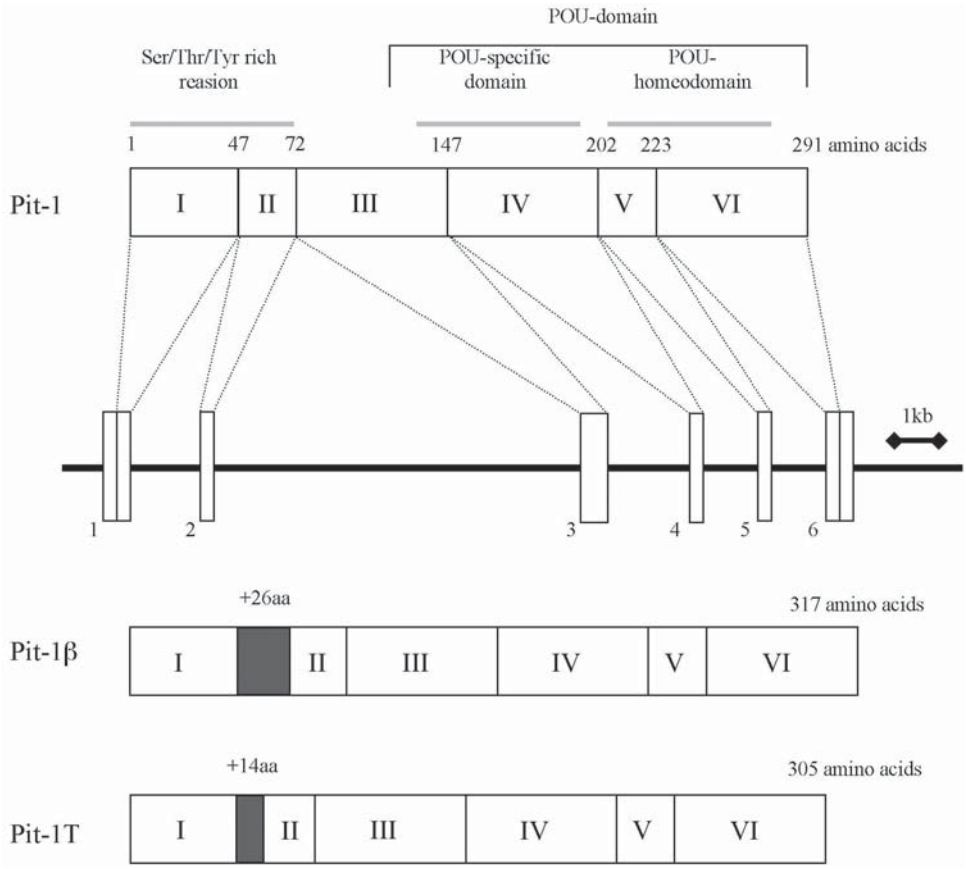


Figure 3 Schemes for *Pit-1* molecule and its isoforms.

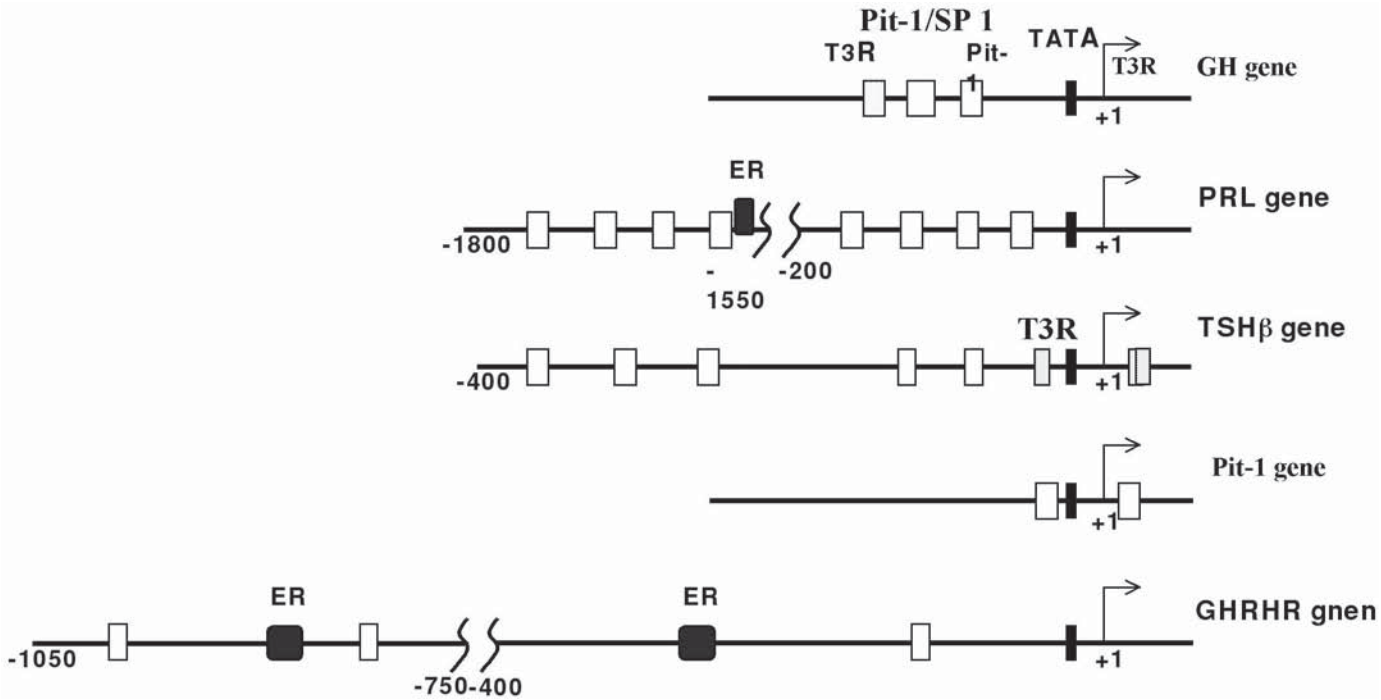


Figure 4 *Pit-1* binding sites in *GH*, *PRL*, *TSH*, *Pit-1*, and *GHRHR* genes. □, *Pit-1* binding site; ■, estrogen receptor binding site; ◫, T3 receptor binding site.

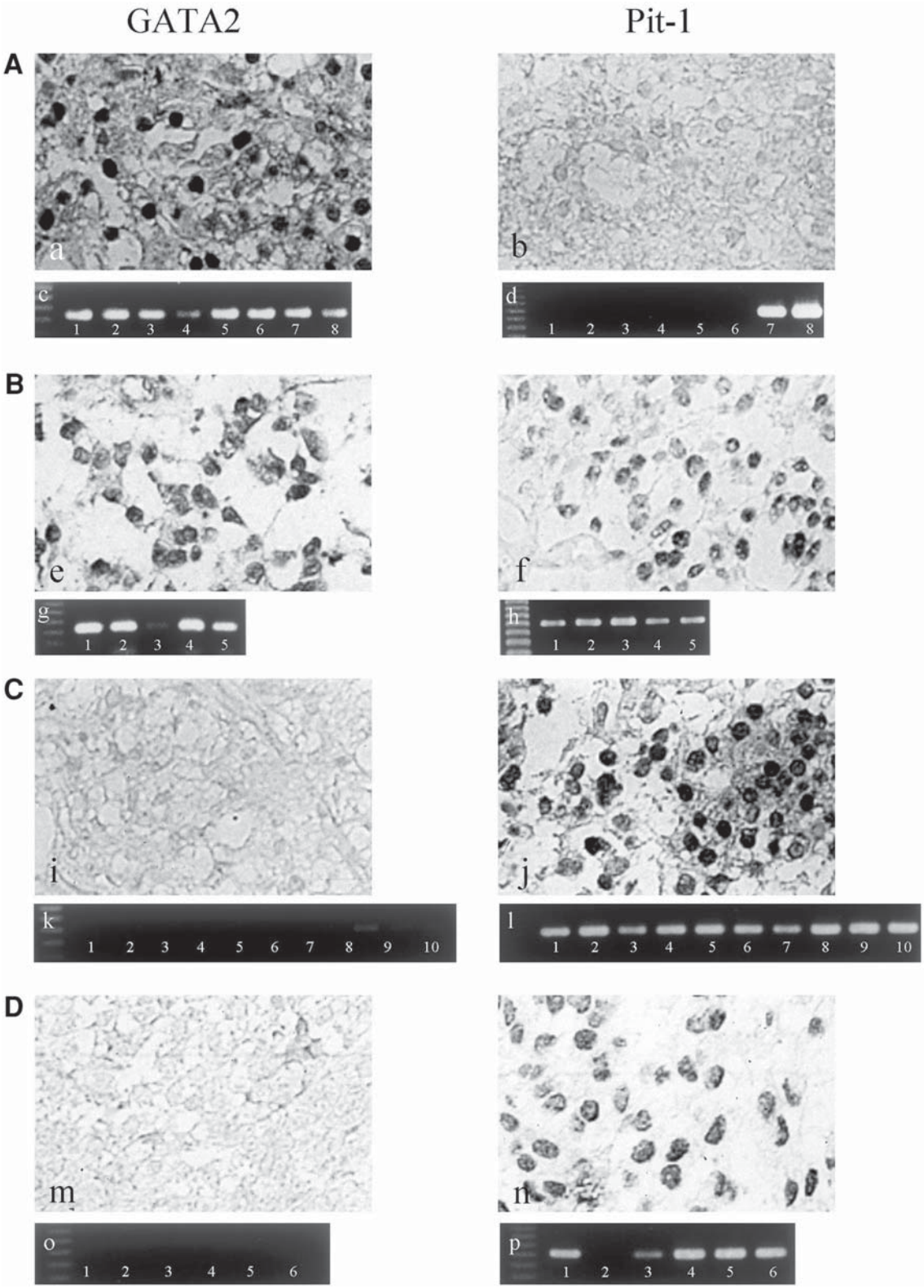


Figure 5 The expression of GATA-2 and Pit-1 in human pituitary adenomas by immunohistochemistry and RT-PCR. The expressions of GATA-2 are shown in the left column and the expressions of Pit-1 are shown in the right column. In gonadotropin-subunit (Gn)-positive adenomas (**A**), GATA-2 protein is detected in the nuclei (**a**) and GATA-2 mRNA is detected in all Gn-positive adenomas (**c**), but Pit-1 protein is not detected in the adenoma (**b**), Pit-1 mRNA is detected in two of eight Gn-positive adenomas (**d**). In TSH-secreting adenomas (**B**), both of GATA-2 and Pit-1 are expressed in all TSH-secreting adenomas (**e** and **f**: immunohistochemistry; **g** and **h**: RT-PCR). In GH-secreting adenomas (**C**), GATA-2 protein is not detected (**i**) and GATA-2 mRNA is detected in 3 of 10 GH-secreting adenomas (**k**). Pit-1 protein and mRNA are expressed in all GH-secreting adenomas. In PRL-secreting adenomas (**D**), neither GATA-2 protein (**m**) nor mRNA (**o**) is expressed, while Pit-1 protein is expressed in the nuclei (**n**) and Pit-1 mRNA is expressed the cytoplasm in five of six PRL-secreting adenomas (**o**).

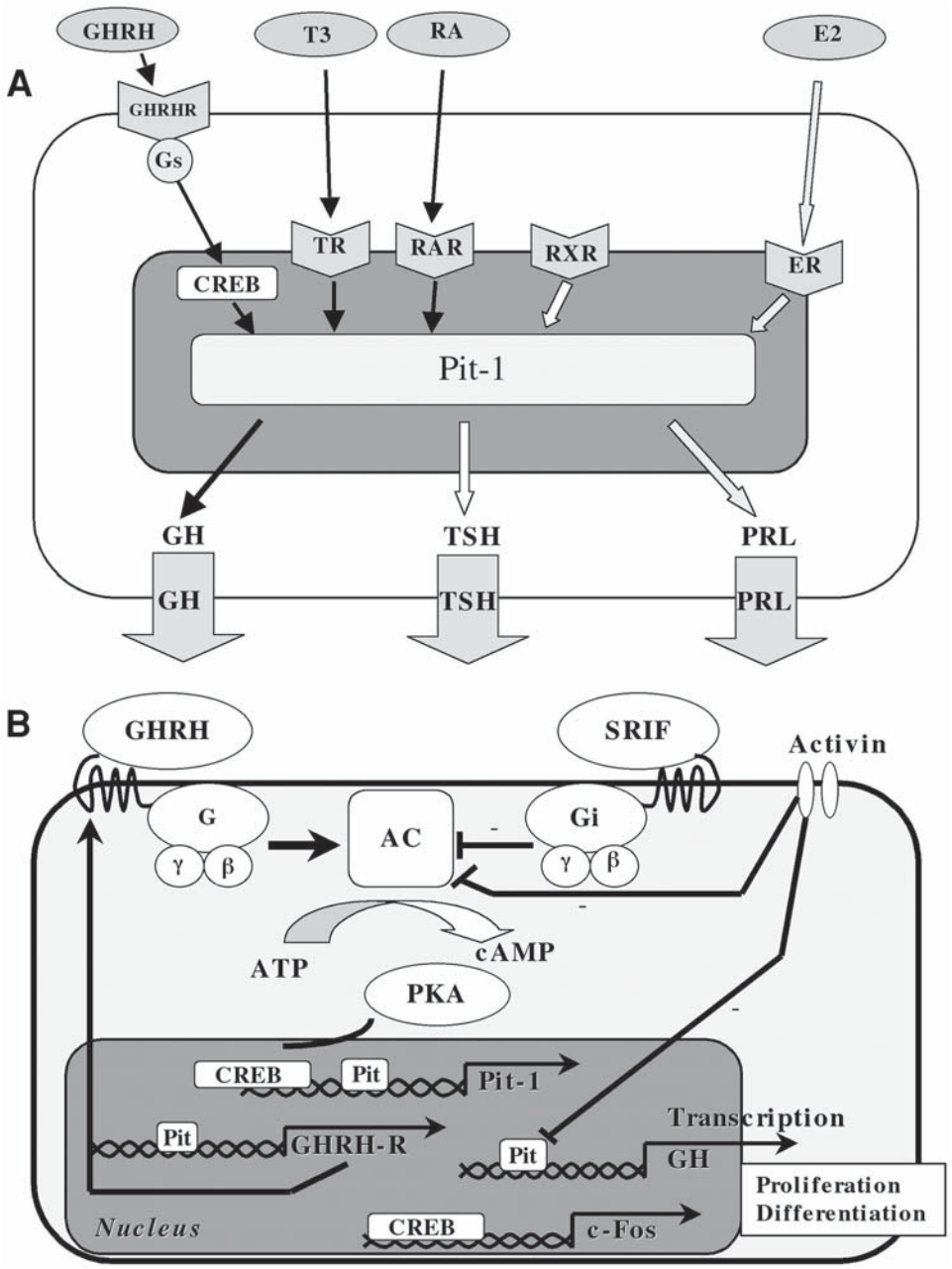


Figure 6 *Pit-1* function. (**A**) Hormone receptors synergize with *Pit-1* activation. Various signals through the respective receptors activate *Pit-1* and each hormone, GH, PRL, and TSH, that is controlled by *Pit-1*. (**B**) In GH-secreting cells, *Pit-1* functions in GH transcription. In GHRH binding GHRH-R and *Pit-1* activate GH secretion. *Pit-1* activates itself with an ATP signal.

The prophet of *Pit-1* (*Prop-1*) has been isolated by a positional cloning in the Ames dwarf mouse (*df*) which is located on chromosome 11 [10,11]. The *df* mutation is associated with depletion of GH, PRL, and TSH cells. It has been demonstrated that *Prop-1* is capable of early enhancement of *Pit-1*. In humans, it has been observed that mutations of *Prop-1* cause combined pituitary hormone deficiency (CPHD), which is a disorder resulting from an impaired pituitary function characterized by impaired production of GH and other pituitary hormones [12]. The CPHD is presented with GH, PRL, TSH, and gonadotropin deficiency. Recently, a concomitant alteration of corticotroph function has been described [13]. Some mutations of *Prop-1* in humans are reported as a homozygous C to T transition (*C217T*) in exon 2, an intronic point mutation (A to T substitution) in exon 3, and a 2-bp GA deletion (*296delGA*) [12–15].

GHRH-R is a receptor for GHRH that is produced and secreted in the hypothalamus. GHRHR is a seven-transmembrane domain receptor with molecular mass of 47 kDa (Fig. 4B) [16]. *Pit-1* biding sites are present in the upstreams of not only *GH*, *PRL*, and *TSH* genes, but also GHRHR and even *Pit-1* genes (Fig. 6B) [17].

MUTATIONS OF OTHER TRANSCRIPTION FACTORS

Lhx3 and *Lhx4*, *LIM* homeobox gene, and Rathke's pouch homeobox (*Rpx, Hesx1*) appear as the earliest known factors for

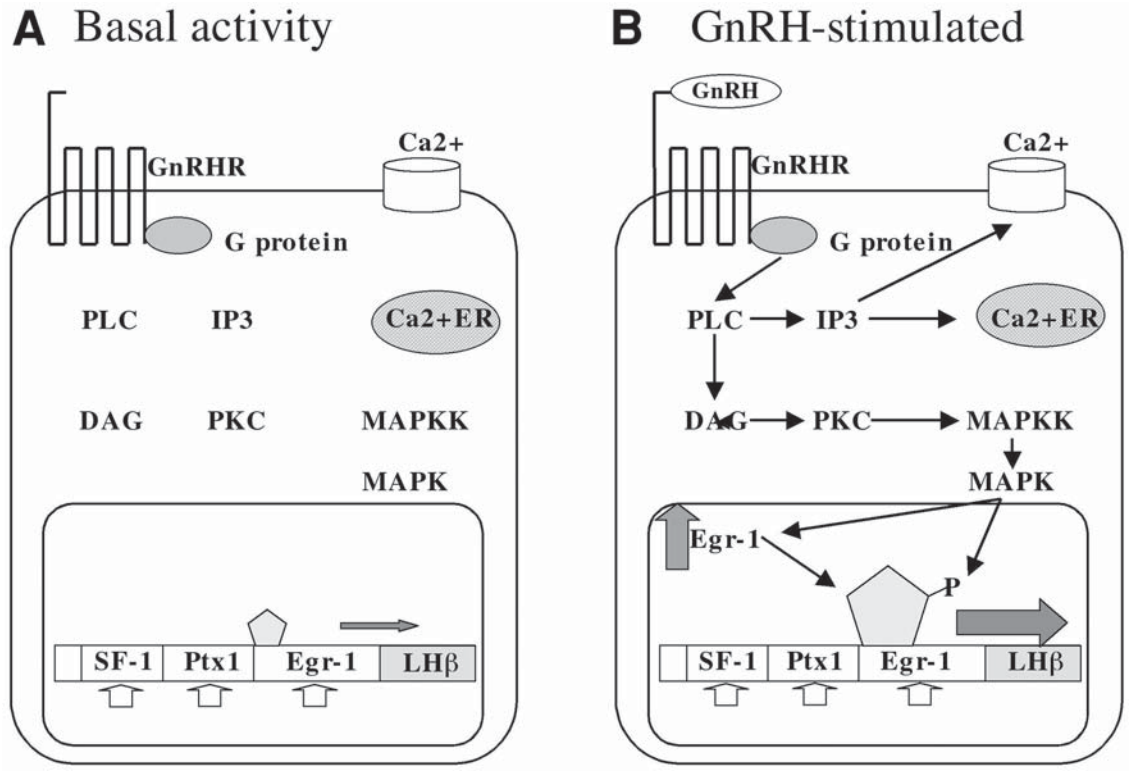


Figure 7 *Egr-1* synergizes with *Ptx1* and *SF-1* to increase rapidly LH-β gene expression. (**A**) In the absence of GnRH, *Egr-1* expression is low. The *LH-β* gene is activated by *Ptx1, SF-1,* and *Egr-1*. (**B**) When GnRH binds to its receptor (GnRH-R), *Egr-1* is activated and its synergizes with *Ptx1* and *SF-1*. ER, Endoplasmic reticulum; MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase.

Rathke's pouch [18–20]. *Rpx* mutations in mice are known as two types: class I mutants showed an absence or substantial reduction of telencephalic vesicles, eyes, olfactory placodes, and frontonasal mass and in contrast class II mutants exhibited less severe craniofacial dysplasia and frequently only one eye was affected. In both mutants, the anterior pituitary is small. Fibroblast growth factor-8 (*Fgf-8*) expressed in the ventral diencephalon is reduced significantly in at embryonic day 8.5. The analysis of *Shh* (Sonic hedgehog), *Nkx2.1* (a member of the vertebrate *Nkx* family of homeobox genes), and *Pax6* (a member of the mammalian *Pax* transcription factor family), which are markers to determine the dorsoventral patterning of the neural tube, indicates that *Shh* is not expressed in class I mutants and is normally expressed in class II mutants. *Nkx2.1* is absent in some mutants, *Pax6* is expressed the same in mutants and wild-type. The mutants also indicate an association with septooptic dysplasia (SOD) [21]. In screening of human SOD patients, some mutants have been discovered: the *C473T* mutant which results in the loss of the *Cac8I* restriction site, the *A374G* substitution in exon 3, the *C509T* missense mutation, the *A541G* mutant, and the *G18C* mutant. In *C509T*, *A541G*, and *G18C* mutants, GH deficiency is exhibited, and in the *G18C* mutant, TSH and LH/FSH deficiencies are also exhibited [21,22].

In animals with $Lhx3^{-/-}$ or $Lhx4^{-/-}$, the oral ectoderm invaginates to form Rathke's pouch and in animals with double knockout of both $Lhx3^{-/-}$ and $Lhx4^{-/-}$, the invagination occurred but resulted in a pouch rudiment [19]. The pituitary development in these mutant animals showed that Rathke's pouch gives rise to a pituitary structure in the presence of at least one copy of *Lhx3*, but not in the absence of *Lhx3*. In the pituitary of the null *Lhx3* mutation ($Lhx3^{-/-}$), GH, TSH-β subunit, α-SU, LH, and *Pit-1* are deficient, but POMC is expressed in not only the pituitary but also in the floor of the diencephalon. Although POMC is expressed, it is drastically reduced and confined to a small cohort of cells in these animals. On the other hand, in the pituitary of the null *Lhx4* mutant, α-SU, GH, TSH-β subunit, and *Pit-1* are present. However, only a few LH-β α-subunit-positive cells and a few GnRH-R-positive cells are present. Furthermore, in these mutant animals, cell proliferation of the anterior lobe is affected, the anterior lobe is hypoplastic, and the numbers of all five hormone-secreting cells are reduced. Recently, human *Lhx3* has been cloned and mapped [23]. In humans, two mutations of Lhx3 have been discovered. One is a homozygous A to G transition that results in a *Y116C* substitution and the other is a homozygous deletion of 23 bp and the adjacent splice donor site. In these cases, pituitary hormones are completely deficient except for POMC [23]. The phenotype of these cases is similar to the phenotype of the aforementioned *Lhx3* mutant in mice.

MOLECULAR TECHNIQUES TO STUDY MOLECULAR MECHANISMS

IMMUNOHISTOCHEMISTRY IHC has been a key technique for the detection of proteins. Recent developments of more sensitive methods including dextran polymer technology and tyramide signal amplification have enabled investigators

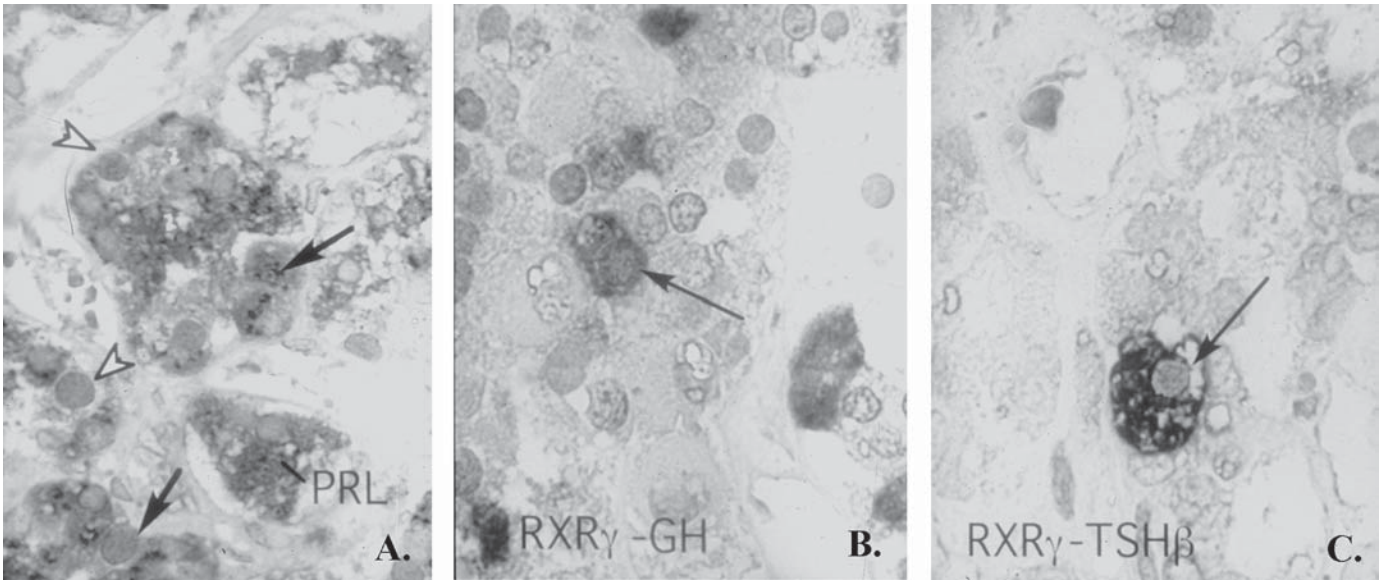


Figure 8 Immunohistochemical co-localization of transcription factors (ER, RXR, and NeuroD1) (brown) and pituitary hormones in rat pituitary (blue). Each transcription factor was detected in the nuclei of anterior lobe. ER was localized in the nuclei of PRL-secreting cells (**A**). RXR was localized in the GH (**B**) or TSH-secreting cells (**C**). (Color illustration appears in insert following p. 148.)

to detect small amounts of proteins such as cell membrane receptors and cytokines. ISH detects mRNA in cells and tissue preparations. The preceding sensitive methods for IHC also can be applied for ISH to detect trivial amounts of mRNA.

Reverse transcriptase-polymerase chain reaction (RT-PCR) is a frequent molecular technique that detects from cell extracts small amounts of mRNA. *In situ* RT-PCR is another state technique that allows one to detect very small amounts of mRNA in specific cells in the tissue sections. Laser capture microdissection (LCM) is sometimes combined with RT-PCR analysis of mRNA or DNA from single cells or well-defined groups of cells. The analysis of DNA by this method is at times essential to study mutation of certain genes.

GENE TARGETING Gene targeting, which includes the production of transgenic and knockout animals, is also used to study the function of certain transcription factors or cofactors. GHRH transgenic mice or rats have been successfully used as a model of GH-secreting pituitary tumors (Fig. 8) [24]. SF-1 knockout mice in the anterior pituitary have shown hypogonadotropic hypogonadism with sexual infantilism, sterility, and severe gonadal hypoplasia [25].

CELL LINES Several cell lines have been established and used extensively for the study of detailed molecular mechanisms. These cell lines include αT3-1 and LβT-2 as indicated in Fig. 6. These cell lines correspond to different developmental stages of various cell lineages (Fig. 6). Rat MtT/S cells have been known as GH-producing cells. GH3 [26] and MtT/SM cells have been known as GH- and PRL-secreting cell lines. The MtT/E cell line is a nonfunctioning cell line derived from the estrogen-induced mammotropic pituitary tumor (MtT) as well as MtT/S and MtT/SM cell lines [27]. The MtT/E-2 cell line established from MtT/E cells is proliferated by estradiol stimulation and the cells secrete GH [28]. These cells are in the GH- and PRL-secreting cell line cells and express *Pit-1* mRNA. Mellon et al. have immortalized and established a few familiar cell lines (gonadotropes and thyrotrope cells)—αT1-1, αT3-1, LβT2, TαT1 [29,30]. αT1-1 cell line, which produces only α-SU, is an early progenitor cell line. αT3-1 cells produce α-SU and express *SF-1* and GnRH-R; LβT2 cells produce LH-α and β-SU and express *SF-1* and GnRHR. In αT3-1 cell lines, GnRH stimulation and increases GnRH-R and α-SU. The cell-specific promotion of the function of αT3-1 cells lies in the binding of SF-1 and AP-1 as well as the GnRHR-activating sequence (GRAS) element [31]. αT3-1 cells possess activin and the activin receptor. TαT-1 cells produce TSH-α and β-subunits, and express *Pit-1*. AtT-20 cells produce POMC and express *Ptx1*. Lloyd et al. have established the cell line HP 75 from a human nonfunctioning plurihormonal adenoma and these cells produce FSH-β and LH-β subunits and express α-SU [32]. The cell line has been maintained for more than 4 yr and should be useful for many investigative questions.

SPECIFIC ASPECTS OF HUMAN PITUITARY DEVELOPMENT AND PITUITARY ADENOMAS

As briefly mentioned previously, the human pituitary is unique in various aspects such as fetal presence of the intermediate lobe and the concomitant production of α-SU in GH-producing cells. "Invasive anterior cells" in the posterior lobe are also a unique counterpart of the distinct intermediate lobe in rodents [1,2].

Pituitary adenomas are infrequent intracranial tumors in humans and are classified according to their function—GH-secreting, PRL-secreting, TSH-secreting, ACTH-secreting, FSH-secreting, and nonfunctioning (NF) adenomas. Previous investigations, have shown that the functional differentiation of the human pituitary adenomas in general is regulated by transcription factors and a combination with cofactors that regulate normal pituitary cells. Human pituitary adenomas are frequently multihormonal, but usually within a certain cell lineage. Occa-

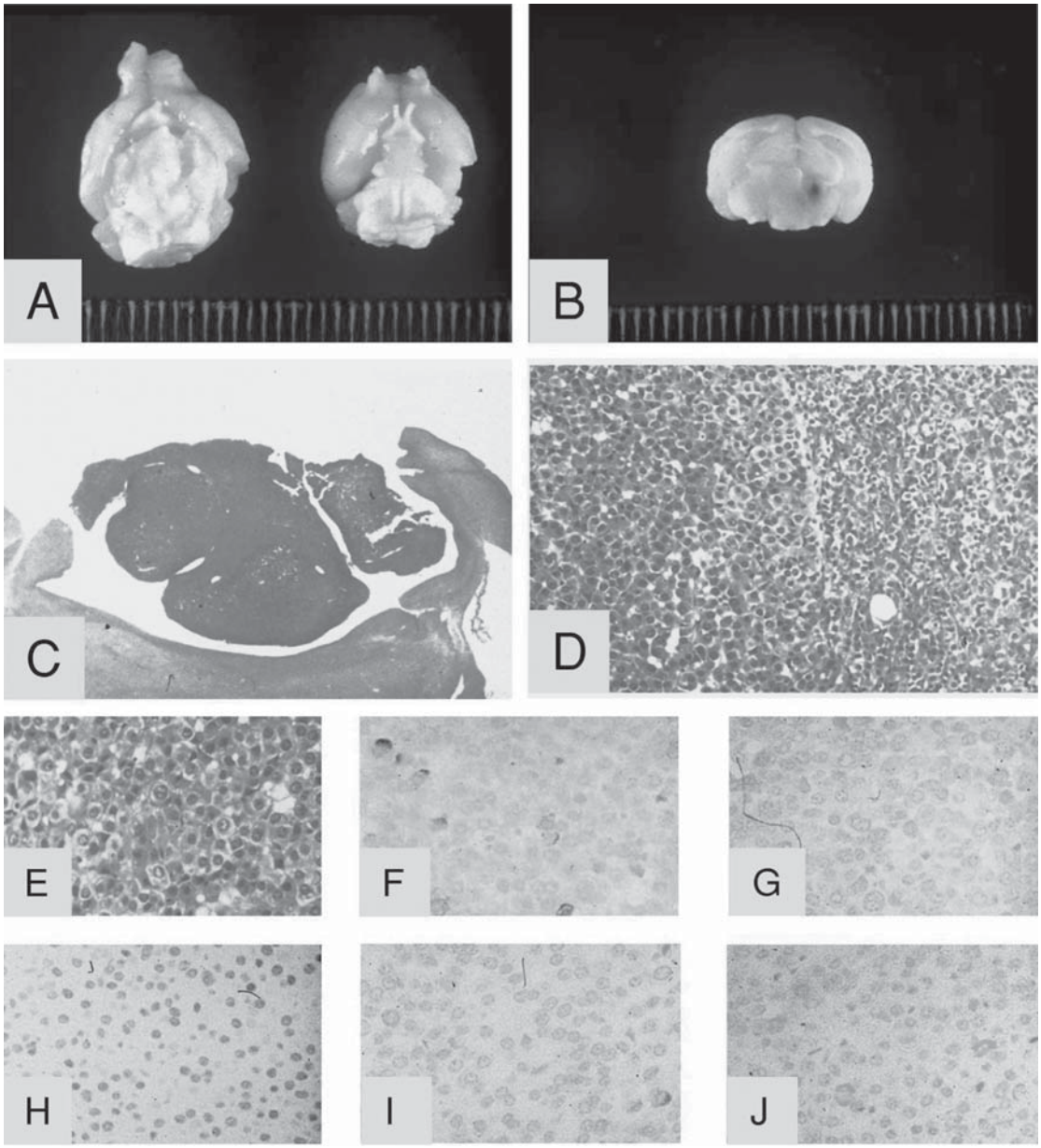


Figure 9 Human GHRH transgenic (Tg) mouse. Ten mice developed pituitary adenomas. A pituitary adenoma in Tg mouse (left) is compared to that of control (right) (**A**). A coronal section of the brain shows a large pituitary tumor (**B**). The results of HE staining of the tumor are shown in panel **C**,**D**,**E**. The pituitary adenoma immunohistochemically express GHRH (**F**), GH (**G**), Pit-1 (**H**), PRL (**I**), and TSH (**J**). (Color illustration appears in insert following p. 148.)

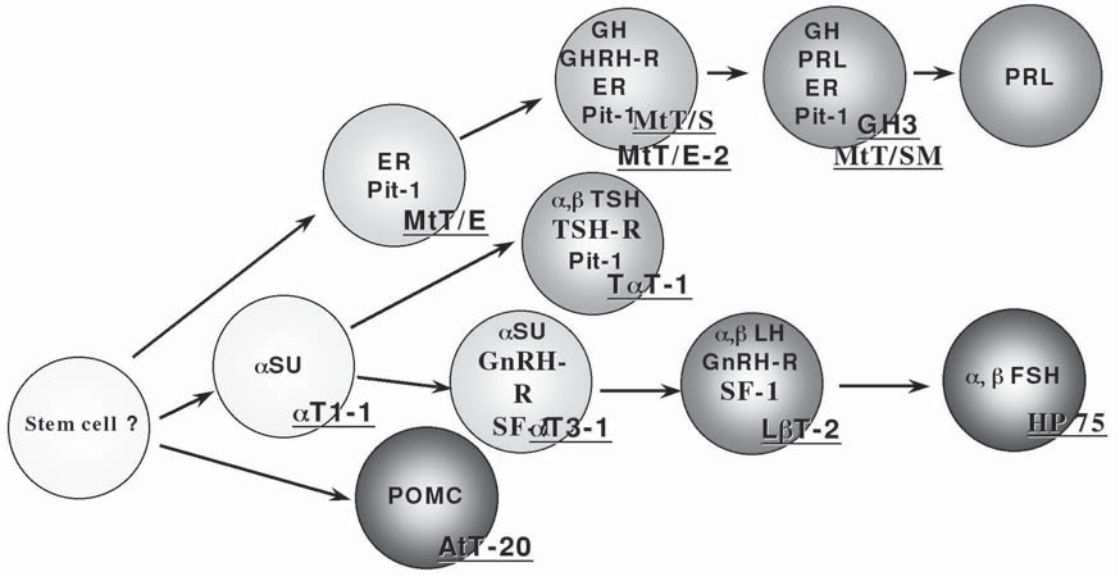
sionally, one adenoma produces multiple hormones belonging to different cell lineages, such as GH and ACTH. It has been found that these tumor cells contain *Pit-1* (Fig. 5) [33] and *NeuroD1* (Fig. 10) [34], which do not cooperate in the normal pituitary cells. This could be considered as an "aberrant" expression of transcription factors in neoplastic conditions (Fig. 11).

TRANSFECTION OF TRANSCRIPTION FACTOR GENE PRODUCES NEW HORMONES IN CELL LINES

When the *Pit-1* gene construct with GFP was transfected into AtT20 cells which produce only POMC-derived peptides and express *Ptx-1* and *NeuroD1* as transcription factors, the transformed AtT20 cells produced not only *Pit-1* protein bound to the nuclei but also GH protein in addition to ACTH. These results were considered to represent a model of the above "aberrant" expression of transcription factor producing new translineage hormones. A similar phenomenon was observed in αT3-1 cells transfected with the *Pit-1* gene, but was not observed with nonendocrine COS-1 cells [35].

SUMMARY

This chapter has emphasized the development and functional differentiation of the anterior pituitary cells, covering the recently disclosed molecular mechanisms involving various transcription factors and cofactors. Various molecular techniques used in the study of pituitary transcription factors were also reviewed.



Hormone	Cell name	origin
GH	MtT/S	rat
GH/PRL	GH3	mouse
	MtT/SM	rat
TSH	TαT-1	mouse
LH/FSH	LβT-2/LβT-4	mouse
αSU	αT3-1/αT1-1	mouse
	HP75	human
ACTH	AtT-20	mouse
FS cell	TtT/GF	mouse

Figure 10 Pituitary cell lines. Each cell line produces individual pituitary hormone and/or hormone receptors.

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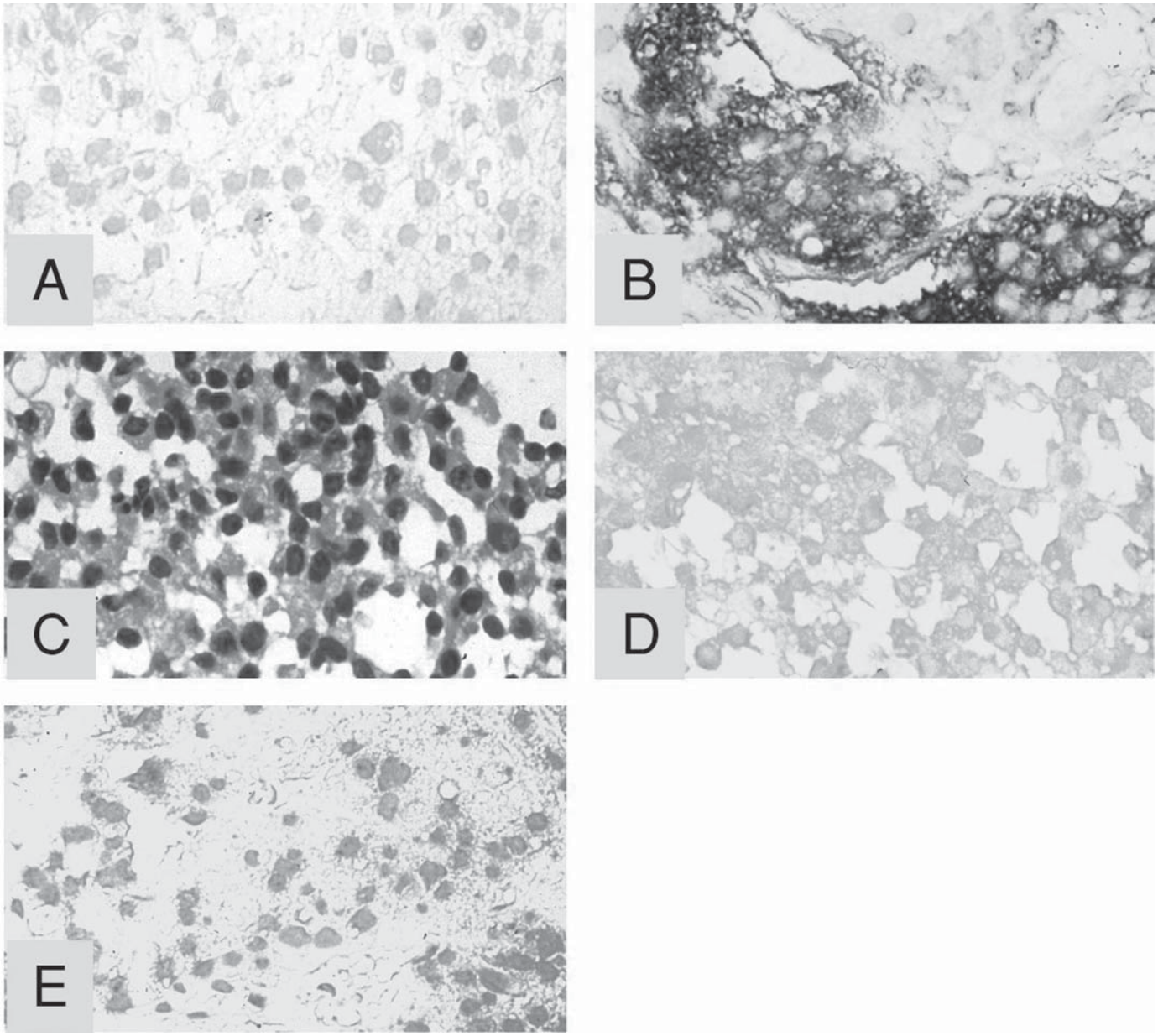

Figure 11 Immunohistochemistry for NeuroD1 in human normal pituitary and ADTH-secreting adenoma. NeuroD1 is observed in the nuclei of both human normal pituitary (**A,B**) and ACTH-secreting adenoma (**C,D,E**). Double staining for Ptx1 (brown) and ACTH (blue) in human normal pituitary is shown in the panel **B**. In human ACTH-secreting adenoma, HE staining is shown in the panel **C**, ACTH is expressed diffusely in the cytoplasm (**D**), and NeuroD1 is also expressed in most nuclei (**E**). (Color illustration appears in insert following p. 148.)

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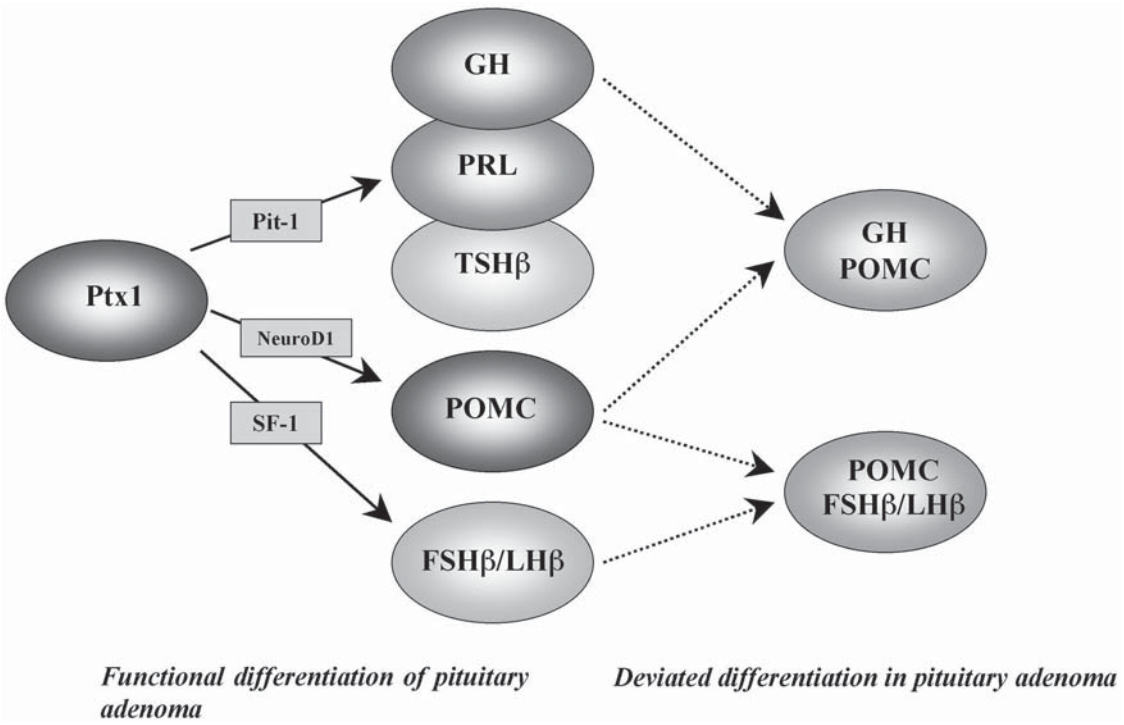

Figure 12 Functional differentiation of pituitary adenomas in relation to transcription factors; lineage oriented differentiation (**left**) and trans-lineage differentiation (**right**).

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5 Recent Developments in the Molecular Biology of Pituitary Tumors

RICARDO V. LLOYD, MD

INTRODUCTION

In recent years, significant advances have been made in understanding the molecular mechanisms regulating pituitary tumor growth and development [1–4]. Various studies have shown that most pituitary tumors are monoclonal proliferations [1,2] and that tumor development is related to defects in oncogenes and tumor suppressor genes. A growing list of oncogenes and tumor suppressor genes have been implicated in pituitary tumor development (Tables 1 and 2). However, most of the genetic abnormalities involved in the development of these tumors have not been uncovered as yet.

ONCOGENES

***Gsp* GENE** The *Gsp* gene is an oncogene located on chromosome 20 and is a mutant form of the α-subunit of Gs transmembrane signaling protein. Gs_α is associated with a dominant activating mutation and has been observed in some growth hormone tumors [3,4] and Table 1). Mutations in the *Gsp* oncogene occur mainly in growth hormone tumors, and the frequency of mutations varies with geographic location. In Japan, fewer than 10% of patients with growth hormone tumors have *Gsp* mutations, while reports from Korea and Europe indicate that up to 40% of patients have these mutations [5,6]. The constitutively activated cAMP-responsive nuclear transcription factor CREB might be promoted by Gs_α overexpression, and this may contribute to growth hormone tumor development [7].

***Ras* GENES** There are three functional genes: H-, K-, and N-*ras*. These are membrane anchor G proteins, and are located on chromosome 5p13. Mutations in H-*ras* have been reported in metastatic pituitary carcinoma and in rare aggressive prolactin adenomas [8–10]. These findings suggest that H-*ras* may be a late event in human pituitary tumor development.

PITUITARY TUMOR TRANSFORMING GENE (*PTTG*) The *PTTG* gene was recently cloned and characterized [11]. The human *PTTG* family consists of at least three homologous genes. *PTTG1* is located on chromosome 5q33. The levels of this gene are low in normal human pituitary, and there is a 60% increase of *PTTG* expression in pituitary tumors. Some tumors have more than a 10-fold increase in *PTTG* expression [12]. PTTG may be a new marker of invasiveness in secretory pituitary tumor [12].

CYCLIN D1 Cyclins and cyclin-dependent kinases (CDKs) are essential for regulation of cell cycle progression in eukaryotes. Active cyclin–CDK complexes promote cell progression through the checkpoints of the cell cycle by phosphorylation of the protein substrates essential to progression to the next phase of the cell cycle. The D cyclins include D1, D2, and D3. These have been mapped to chromosomes 11q13, 12p13, and 6p21, respectively. *Cyclin D1* has been shown to be a protooncogene, and *cyclin D2* may have similar functions [13,14]. *Cyclin D1* (*CCND1*) is often amplified in human tumors such as breast cancer. The cyclin D1 protein is coded for by the *PRAD1–CCND1* (*Bcl-1*) gene, and many cancers have amplifications of the 11q13 chromosome band. Allelic imbalance of the *cyclin D1* gene has been observed in pituitary tumors, and this has been associated with the more invasive tumors [15]. Immunostaining for D cyclins in pituitary adenomas has been variable. Some studies have shown nuclear staining in 20% of tumors, while cytoplasmic staining was present in 35% of tumors [15]. The significance of cytoplasmic staining is uncertain for the D cyclins. There is usually no correlation between *cyclin D1* positivity and tumor grade or between allelic imbalance and expression of this protein [15]. It has been suggested that *cyclin D1* expression and alterations occur early in pituitary tumor development and may be more frequently associated with nonfunctional tumors. One study has shown only sparse staining for *cyclin D1* in pituitary tumors with nuclear immunoreactivity more common in nonfunctioning and aggressive tumors compared to functioning tumors and normal pituitary [16]. In a study of rodent pituitaries [17] and human pituitary tumors, very little cyclin D1 protein was expressed, while cyclin D3 was expressed by both functional and nonfunctional tumors [18]. Cyclin D2 was more commonly expressed by nonfunctional than functional tumors in a study of human pituitary adenomas [18].

TUMOR SUPPRESSOR GENES

Many tumor suppressor genes have been associated with pituitary tumor development in human and experimental animals.

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Table 1
Putative Oncogenes Involved in Pituitary Tumorigenesis

Oncogene	*Comments*
Gsp	Gs protein gsp mutations in 10–40% GH tumors [3,4]
PTTG	Pituitary tumor transforming gene overexpressed in many pituitary tumors [11,12]
Cyclin D	*Cyclin D1* (*CCND1*) gene shows allelic imbalances in some tumors. *Cyclin D2* and *D3* abnormalities may also be significant [13–16]
CREB	Constitutively activated cAMP-responsive nuclear transcription factor. *CREB* may facilitate GH cell transformation [7].
Ras	H-*ras* mutations detected in metastases from pituitary carcinoma [8,9]

Table 2
Putative Tumor Suppressor Genes Involved in Pituitary Tumorigenesis

Suppressor gene	*Comments*
MEN1	Multiple endocrine neoplasia 1 (*MEN1*) gene, menin, may have a limited role in the pathogenesis of sporadic pituitary tumors [19,20]
Rb	Loss of Rb expression seen in some animal pituitary tumors, but mutations uncommon in human pituitary tumors [21,22]
p53	Overexpression of p53 protein common in pituitary carcinomas, but mutations infrequent [23,24]
nm23	Reduced expression seen in invasive adenomas in one study [27]
ZAC	Expression reduced in null cell and gonadotroph tumors compared to normal pituitaries [28]
GADD45γ	Expression reduced in nonfunctioning adenomas compared to normal pituitaries [29]
p16/CDKN2A	Hypermethylation frequent in some subtypes of pituitary tumors compared to normal pituitaries [38]
p27	Knockout mice develop pituitary intermediate lobe tumors. Human pituitary tumors have decreased levels of p27 protein expression especially in ACTH tumors. Mutations not found [39–57].
p18	Knockout mice develop intermediate lobe hyperplasia and tumors. Double knockout mice ($p27^{-/-}/p18^{-/-}$) rapidly develop pituitary intermediate lobe tumors and die from these in 3–4 mo. Status in humans not currently known [58,59]

Some of these genes may not be associated with classical genetic mutations or gene alteration, which is usually a common feature of suppressor genes in many tumors.

***MEN1* GENE** The *MEN1* gene, which is located on chromosome 11q13, has been associated with germline mutations in many familial and in a few sporadic multiple endocrine neoplasia type 1 (MEN1) patients [19]. Few patients with sporadic pituitary tumors have mutations or other alterations in the *MEN1* gene [20]. This suggests a limited role of this gene in pituitary tumor development and pituitary tumor progression (Table 2).

RETINOBLASTOMA SUSCEPTIBILITY GENE (*Rb*) Mice with knockout of the *Rb* gene, located on chromosome 13q14 in humans, frequently develop tumors of the pituitary intermediate lobe. However, mutations of the *Rb* gene are uncommon in human pituitary tumors [21]. In one study, Simpson et al. found *Rb* expression in 3 growth hormone and 53 nonfunctional tumors, and 27% of growth hormone tumors and 4% of nonfunctional tumors did not express *Rb* proteins [22].

***p53* GENE** The *p53* gene, located on chromosome 17, is the most commonly altered gene in human cancers. However, genetic alterations in *p53* are uncommon in human pituitary tumors [23,24]. Overexpression of *p53* protein has been reported commonly in pituitary carcinomas [25,26]. This overexpression may be related to alterations in *p53* synthesis or processing in the tumor. The basic mechanisms leading to *p53* overexpression in pituitary tumors is currently unknown.

nm23 The *nm23* gene is located on chromosome 17q24-q25. It has a purine factor binding substrate and is present in a wide spectrum of metastatic tumors including those in colon, breast, and liver. One report indicated that the nm23 protein hybrid showed reduced expression in invasive pituitary tumors [27]. Genetic mutations were not associated with this decreased expression. More studies are needed to clarify the role of nm23 in pituitary tumor development and progression.

ZAC The *ZAC* gene encodes a new zinc finger protein that induces apoptosis and cell cycle arrest. It is localized on chromosome 6q24-q25. *ZAC* is highly expressed in the normal anterior pituitary gland, and use of antisense experiments promote pituitary cell proliferation. In a recent study, *ZAC* expression in pituitary tumor was analyzed, and there was decreased or absent *ZAC* mRNA in protein expression in nonfunctioning pituitary tumors (gonadotrophs and null cell adenomas). In clinically functional pituitary tumors, there was a variable decrease in *ZAC* expression. Mutations in the *ZAC* gene were not noted in these tumors with loss of expression. These findings suggest that there are other mechanisms of *ZAC* gene inactivation in pituitary tumors [28].

GADD45γ *GADD45*γ, which is also known as cytokine response 6, is a *p53*-regulated human gene involved in growth suppression in apoptosis. It is located on chromosome 98. A recent study of *GADD45*γ expression in pituitary tumors showed that the mRNA was highly expressed in normal human pituitary tissues with loss in nonfunctioning pituitary tumors (17 of 18 cases) [29]. The *GADD45*γ gene was not expressed in most growth hormone or prolactin-secreting pituitary tumors [29]. This study showed that *GADD45*γ expression was lost in the majority of human pituitary tumors. However, mutations of the *GADD45*γ gene have not been reported in pituitary tumors.

p16/CDKN2 p16 or CDKN2A is a cell cycle protein and belongs to the INK family. This family also includes p15/INK4B, p18/INK4C, and p19/INK4D. These proteins have four ankyrin repeats and form complexes with CDK4 and/or CDK6. They also interact with D-type cyclins. Functional activities of the p16 protein is that they interact with the retinoblastoma protein [30]. The *p16* gene is located in chromosome 9p21 and is mutated in some tumors such as melanoma [31], supporting its role as a classical tumor suppressor gene. Woloschak et al. reported *p16*

Table 3
Comparative Genomic Hybridization Analyses of Human Pituitary Adenomas

Reference	*Tumor types*	*N*	*Abnormality*	*Chromosomal abnormalities*
[61]	Nonfunctioning	23	74%	Sex chromosome and 18 (34.7%); amplifications of 4q, 5q, 9p, 13q, and 17q (10–30%)
[62]	PRL, GH, TSH, ACTH, nonfunctioning	52	48%	11, 7, X, 1, 8, 13, 5, 14, 2, 6, 9, 10, 12, 3, 18 (decreasing frequency); functional tumors > nonfunctioning tumors
[63]	GH	10	80%	Gains 5, 9, 22q, 17p12 (20–50%); losses 13q, 18 (20–30%)
[64]	PRL, GH, nonfunctioning	12	—	Loss 13q most common (5 cases)
[65]	All types	75	45.3%	Gains 4.9 times more frequent than losses Gains x (32%), 19 (16%), 12 (6.7%), 7, and 9 (6.7%) Loss 11 (5.3%), 13, and 10 (4%)
[66]	Nonfunctional [26] Functional [12]	38		Gains 3, 7, 14, 6p, and 20q

N = number of case studies.

gene alterations in pituitary tumors. They observed that the gene was hypermethylated in some tumors compared to normal pituitary [32,33]. Simpson et al. confirmed these findings and showed that specific subtypes of pituitary tumors, namely, nonfunctional tumors, were hypermethylated, while growth hormone tumors were not [34–37]. Frost et al. used a mouse pituitary cell line (ATT/20) and showed that transfection of the *p16/CDKN2A* gene was associated with a reduction in cell proliferation, indicating, the *p16* could mediate cell growth arrest [38].

p27/Kip1 Another family of CDKI includes the Kip/CIp proteins, which include p27/Kip1 (p27), p21/WAF1/Cip1 (p21), and p57/Kip2 [p57]. These proteins inhibit kinase activities by preactivated G1 *cyclin E/CDK2 cyclin D-CDK4/6* and other cyclins. *p27* has been studied extensively [39]. Mice with *p27* knockout gene developed hyperplasia and tumors of the intermediate lobes of the pituitary [40–42]. Many studies in mice and humans have shown that *p27* is probably important in tumor development of pituitary as well as other tumors [43–50]. In the human pituitary, p27 protein decreases from normal pituitary to adenomas and carcinomas, but mutations of the *p27* gene are very uncommon (reviewed in [39]). Our laboratory first reported that ACTH tumors had the lowest levels of *p27* expression [48], and this has been confirmed by other laboratories [51].

Although *p27* is a putative tumor suppressor gene, the infrequency of mutations of this gene suggest other mechanisms of gene silencing. Methylation of the *p27* gene is also infrequent and has been reported only in a subset of pituitary tumor cell lines [49,50]. Other mechanisms regulating *p27* may be altered by genetic changes that probably have not been discovered as yet. Degradation of *p27* is by the ubiquitin proteosome system, which is a common pathway for degradation of many proteins [52–54]. New findings suggest that F-box proteins, which are part of the ubiquitin protein ligase recognition system for *p27* degradation, may be important regulators of *p27* function [55]. The F-box protein SKP2, for example, is important in degradation of a phosphorylated *p27* [56] and JAB1 is also important for *p27* degradation [57]. Additional studies are needed to examine the role of these genes in regulating *p27* function and in pituitary tumor development.

p18 *p18* is a CDKI that has been shown in knockout mice to be important in pituitary tumor development similar to *p27*. These animals developed tumors in the pituitary intermediate lobe [58]. Animals with both *p27* and *p18* knockouts developed massive pituitary tumors as well as hyperplasias and tumors of other endocrine tissues [58]. The pituitary tumors can lead to death of these mice by a few months of age [59]. The role of *p18* and other CDKIs in development of human pituitary tumor is still unexplored.

OTHER GENETIC CHANGES INVOLVED IN PITUITARY TUMOR DEVELOPMENT There are probably many other genetic alterations that contribute to pituitary tumor development. Some reports indicate that deletions in chromosome 9p probably contribute to pituitary tumor development [60]. Alterations in various chromosomes including chromosomes 10, 11, and 13 have also been implicated by comparative genomic hybridization studies [61–66]. Comparative genomic hybridization studies have highlighted the complexity and the extensive alterations of chromosomal loci in pituitary tumors (Table 3) [61–66]. Additional cytogenetic studies have supported some of the CGH findings such as by fluorescent *in situ* hybridization (FISH) [67].

More recent studies have implicated new genes in the pathogenesis of pituitary tumors. One group identified the *HMGA2* gene on chromosome 12q14-15 that led to pituitary tumors in transgenic mice [68]. The *HMGA2* gene was found to be overexpressed in human prolactinomas [69].

DEVELOPMENT OF PITUITARY TUMORS IN ANIMAL MODELS AND IN HUMANS Much more information is available about the development of pituitary tumors in animal models compared to humans. Figure 1 summarizes some of the data from the literature showing some of the genes involved in mouse pituitary tumors including null or knockout and transgenic models [70–74]. Figure 2 summarizes a putative model for development of pituitary adenomas and carcinomas in humans. Some of these genes such as folate receptor, ornithine decarboxylase, and C-met tyrosine kinase were recently discovered to be overexpressed in pituitary tumors from cDNA array data [75] and have only a putative role in pituitary tumor development at this time.

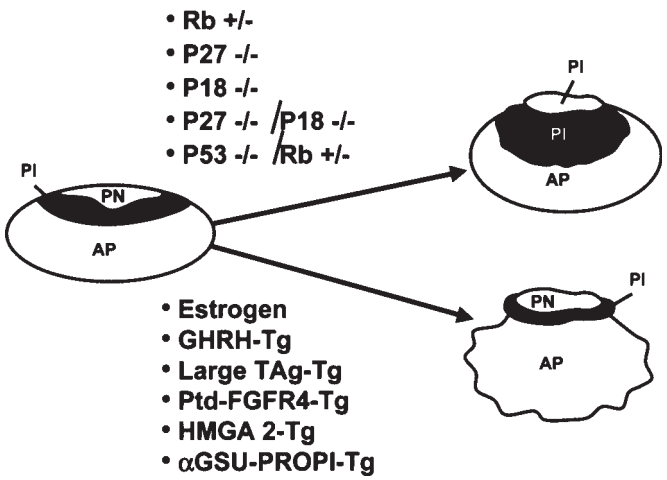

Figure 1 Model summarizing development of pituitary tumors in mice. The pituitary tumor models with knockout or null mice usually lead to tumors of the pituitary intermediate lobes in mice. Many of these genes have included cell cycle regulatory proteins. Hormones such as estrogens lead to prolactin cell hyperplasia and tumors in the anterior pituitary. Transgenic mice such as the growth hormone releasing hormone (GHRH), large T antigen, a variant of fibroblast growth factor receptor 4 (Ptd – FGFR4), and the *HMGA2* gene are associated with tumors in the anterior pituitary. PN, Pars nervosa; PI, Pars intermedia; AP, anterior pituitary; Rb, retinoblastoma gene.

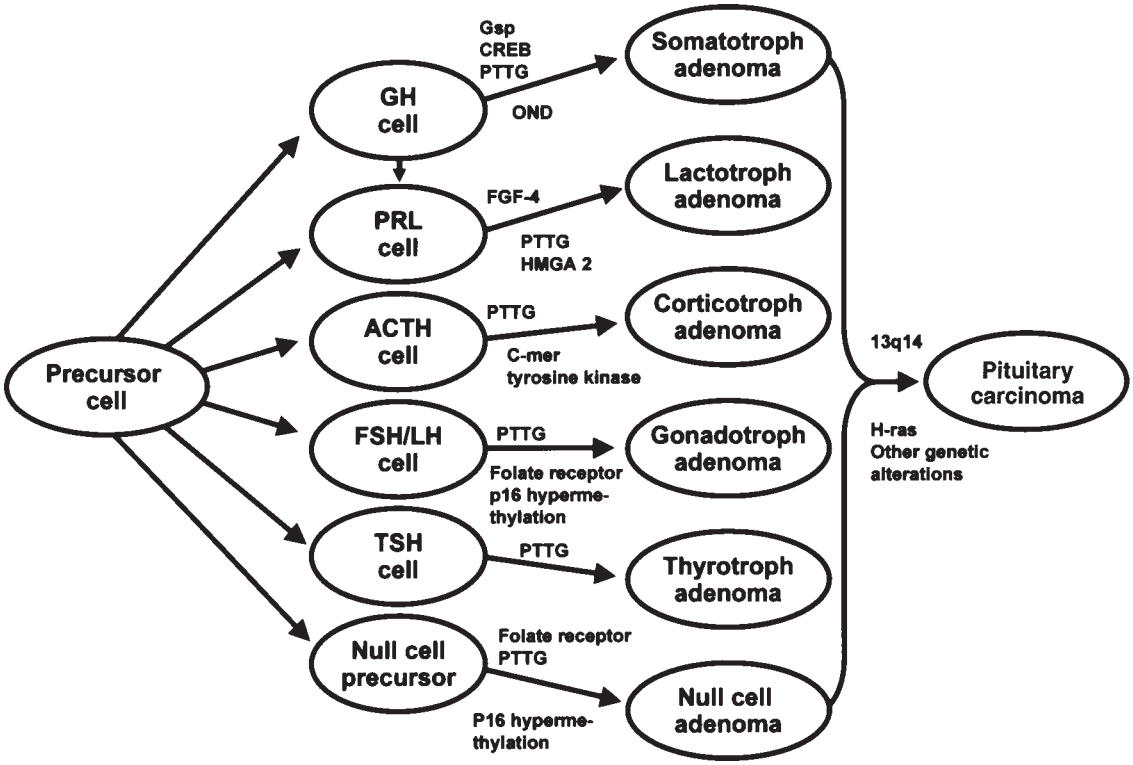

Figure 2 Putative genes involved in the development of human pituitary tumors. GH cells also serve as precursors of most PRL cells. Various types of pituitary tumors have relatively unique genetic alterations leading to adenoma development. Overexpressed genes involved in pituitary tumorigenesis discovered by DNA array technique include ornithine decarboxylase (OND), C-met tyrosine kinase, and folate receptor [75]. Note that very few genes implicated in the development of pituitary carcinomas (defined by metastatic disease) have been discovered to date.

Some generalizations that can be made from these animal models include:

1. Knockout models are usually associated with tumors involving the mouse intermediate lobe of the pituitary.
2. Most transgenic models usually lead to tumor development in the anterior mouse pituitary.
3. Although pituitary transcription factors such as Pit-1 are not associated with pituitary tumors, recent studies have shown that persistent *Prop1* expression enhances pituitary tumor susceptibility [73].

Some generalizations from the human models of pituitary tumor development include:

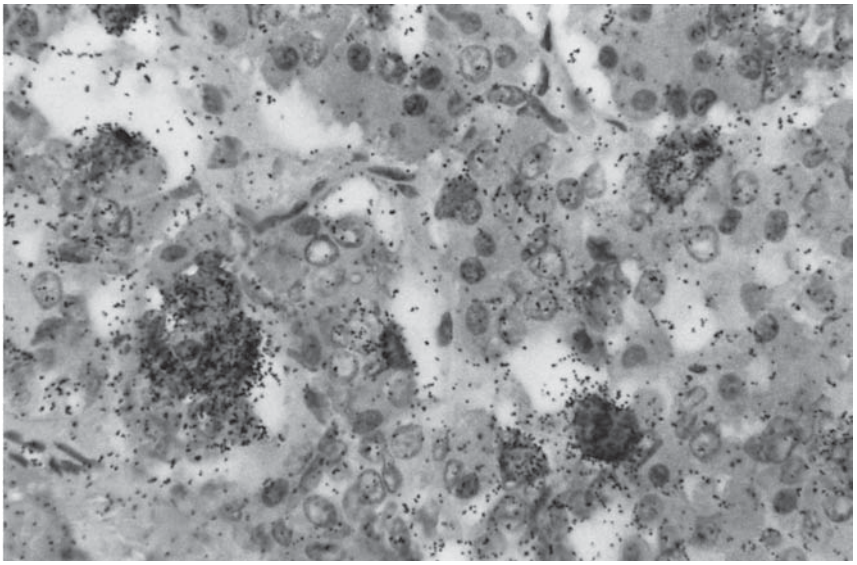

Figure 3 An example of *in situ* hybridization with a radioactive ^{35}S probe localizing POMC cells in a normal pituitary. These cells are also immunopositive for ACTH. Original magnification ×350. (Color illustration appears in insert following p. 148.)

1. Many genes influencing pituitary tumor development in humans are largely cell-type specific. The *Gsp* mutations are predominantly in GH tumors, with *p16* hypermethylation observed mainly in nonfunctional tumors.
2. The genes influencing pituitary carcinoma development in humans are largely unknown. Pituitary carcinomas are defined by the presence of metastatic disease, so aggressive pituitary adenomas would represent an intermediate stage in tumor progression in humans.

HYBRIDIZATION METHODS IN THE STUDY OF THE PITUITARY

Hybridization, which involves paring of complementary strands of nucleic acid such as DNA–DNA, DNA–RNA, or RNA–RNA, is one of the keystones of molecular studies. Various forms of hybridization including solution hybridization, Northern and Southern hybridization, and *in situ* hybridization have provided major insights into molecular mechanisms and disease development. *In situ* hybridization is a powerful technique used in molecular pathology. The relationship of the expression of specific gene products to other cells in the tissue sections or in cell preparations can be readily visualized with this approach. A combination of *in situ* hybridization (ISH) analysis and immunohistochemistry can be used to localize gene transcripts and the translated protein products within the same cell or in adjacent cells.

***IN SITU* HYBRIDIZATION** Various steps are involved in ISH and preservation of nucleic acid is one of the important first steps in the procedure [76–78]. mRNA is better preserved in frozen tissue sections compared to paraffin sections, but reproducible results have been obtained in many studies with paraffin-embedded tissue sections. Fixatives such as paraformaldehyde and neutral buffered formalin are excellent for mRNA preservation. After fixation, tissues can be sectioned and stored for weeks or months without loss of mRNA in the tissues. Many different types of probes can be used for ISH including cDNA and cRNA probes and synthetic oligonucleotide probes. The signal can be detected with radioactive reporters or nonradioactive reporters, which is more commonly used today [79]. Nonradioactive detection is more rapid, but this is usually not as sensitive as a radioactive probe.

HYBRIDIZATION ANALYSIS IN THE PITUITARY

Many studies have been done with ISH studies with human pituitaries (Figs. 3–5). Formalin-fixed paraffin-embedded tissue sections have been used extensively in these studies. One of the earlier studies showed a growth-hormone-secreting pituitary tumor in which the mRNA for growth hormone was present, but the protein was not detected in the cells [80]. Many studies have shown that prolactin-producing cells in tumors commonly express only prolactin but not growth hormone message, supporting the concept that prolactin-producing cells in tumors are terminally differentiated. Other studies have examined the effects of growth hormone releasing hormone and somatostatin on growth hormone gene expression in human pituitary tumors [81,82]. Studies by Kovacs et al. [83] and Trouillas et al. [84] showed that some silent growth hormone tumors without clinical evidence of acromegaly usually express the growth hormone mRNA within these cells. The functional state of the growth hormone messenger in proteins in patients with silent growth hormone tumor is unknown. Other studies [85] examining a series of pituitary tumors from patients with acromegaly compared immunohistochemistry and ISH; there was 100% correlation for growth hormone and 60% for prolactin in the hybridization signal for the hormone content. This finding was in agreement with earlier studies [80]. In other studies of growth hormone and prolactin tumors, investigators [86] found that mRNA for prolactin increased during pregnancy [86]. This finding suggests that a transformation of cell types with the development of mammosomatotroph cells expressing both prolactin

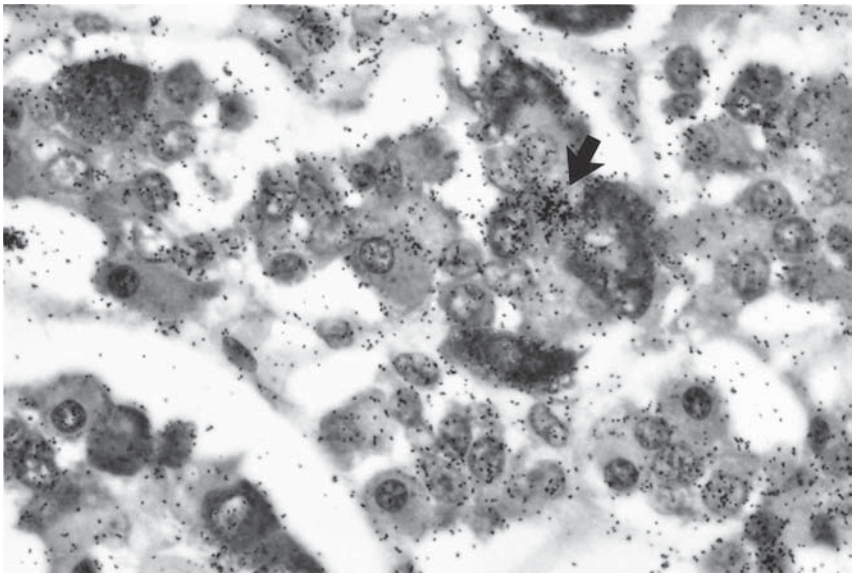

Figure 4 *In situ* hybridization for PRL receptor in the anterior pituitary with a ^{35}S probe. Immunostaining for ACTH shows that the PRL receptor positive cells (*arrow*) are different from the ACTH cells. Original magnification ×350. (Color illustration appears in insert following p. 148.)

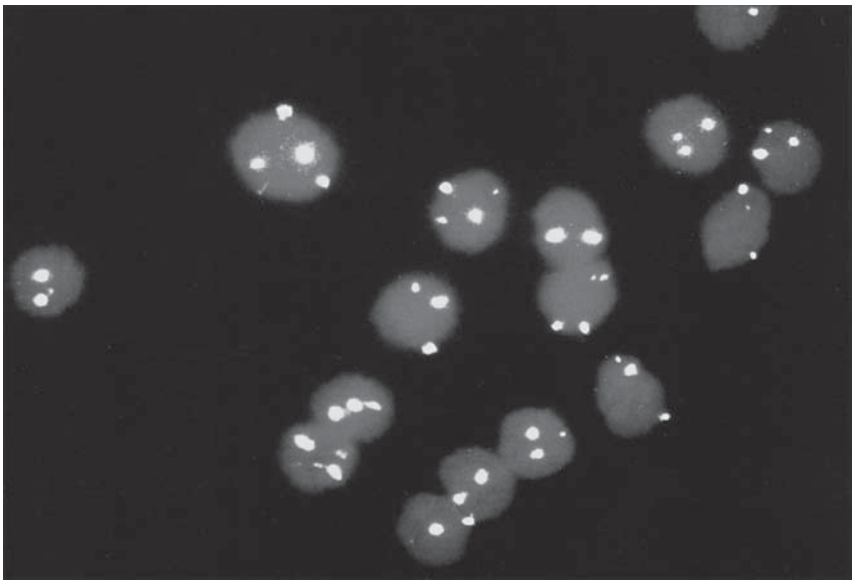

Figure 5 Fluorescent *in situ* hybridization in a smear of pituitary adenoma cells using a probe for chromosome 11 showing trisomy of this chromosome in many of the adenoma cells. Original magnification ×400. (Courtesy of Dr. George Kontogeorgos, Athens, Greece.) (Color illustration appears in insert following p. 148.)

and growth hormone occurred in the altered physiological state of pregnancy. In another study using ISH, investigators reported that prolactin mRNA was decreased by bromocriptine and a population of small cells indicated in these cells responded to the drug with decreased cytoplasmic volume. A subpopulation of the larger prolactin cells did not show a decrease in prolactin mRNA [87].

ISH analysis of ACTH tumors has been done by various investigators [88]. Some studies have shown proopiomelanocortin (POMC) mRNA expression in most functional tumors as well as in some of the silent pituitary tumors. This has been confirmed by other investigators [89,90]. Other studies have found that the silent ACTH tumors may have an abnormal mRNA [91]. Some investigators have used nonradioactive probes to detect POMC mRNA within pituitary tumors [92,93]. Others have shown that in Nelson's syndrome, there was a greater detection of POMC mRNA compared to Cushing's disease, suggesting differences in the transcript expression in these two conditions.

Other investigators have examined non-neoplastic pituitary for POMC gene expression and have shown that POMC mRNA can be detected in postmortem pituitaries up to 66 h after death [94]. Some investigators using quantitative ISH show that there was an increase in POMC mRNA in suicide victims compared to patients who had cardiac death [94,95]. ISH analyses of gon-

adotroph tumors have been reported. The FSH β and LH β genes have been detected in gonadotroph, null cell, and oncocytic tumors [96–99]. This specific finding suggested a close relationship between gonadotroph and null cell tumors. Other studies of gonadotroph, nonfunctional pituitary tumors [100] show that one third of the tumors expressed a β-subunit of human chorionic gonadotrophin. A small number of these tumors also express growth hormone mRNA. Other investigators have found prolactin and POMC mRNA in nonfunctional tumors [97].

A wide variety of other mRNA transcripts have been identified in pituitary tumors by ISH. These have ranged from transcripts for secretory granule protein mRNAs such as chromogranin/secretogranin family to hypothalamic hormones and hormone receptors as well as transcription factors [98–101]. These studies have shown the utility of ISH in analysis of gene expression.

FISH has been used to analyze pituitary chromosomal abnormalities (Fig. 5). These studies provide insight into the pathogenesis of pituitary tumors [67].

IN SITU POLYMERASE CHAIN REACTION ANALYSIS OF GENE PRODUCTS IN PITUITARY TUMORS

Many historical studies used reverse transcriptase-polymerase chain reaction (RT-PCR) for the analysis of gene expression in pituitary tumors [101–106]. In addition to the use of conventional RT-PCR for studies of pituitary tumors, investigators have use ISH and PCR (*in situ* PCR) to visualize low-abundant messages in cells [107–111]. *In situ* PCR studies have usually been done with cultured cells or frozen tissue sections and are more difficult to do with paraffin-embedded tissues for mRNA localization. These studies have localized low copy numbers of genes in specific cellular compartments.

SUMMARY

Although the exact pathogenesis of pituitary tumors is largely unknown, many putative oncogenes and suppressor genes implicated in the development of pituitary tumors have been characterized. New technological developments such as DNA microarrays are contributing to the discovery of new genes involved in the development of human pituitary tumors. More traditional molecular techniques such as ISH and *in situ* RT-PCR have contributed an enormous amount of information about pituitary cell and tumor gene expression when combined with morphological studies of normal and neoplastic pituitaries.

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6 The Pineal Gland

M. Beatriz S. Lopes, MD

THE NORMAL PINEAL GLAND

The human pineal is a small gland shaped as a pine cone and located posteriorly in the midline of the brain, at the quadrigeminal plate cistern. In adults, the gland measures approx 8 mm in its longest diameter and weighs approx 100 mg. During embryonic development, the gland arises from an area of ependymal thickening from the most caudal portion of the roof of the third ventricle that evaginates during the seventh week of gestation [1]. The gland continues to be attached to the third ventricle by a short stalk after birth [2]. However, the pineal gland is not directly connected to the brain in the adult. Its stimulatory pathway appears to consist of the retina and suprachiasmatic nucleus of the hypothalamus (retinohypothalamic tract), the intermediolateral gray column of the thoracic spinal cord, and the superior cervical ganglion [3,4]. The pineal gland is one of the circumventricular organs of the brain. Consequently, it lacks a blood–brain barrier.

Similar to any neuroendocrine gland, the pineal gland is arranged in acini or lobules divided by rich vascular trabeculae. It is basically composed of two cellular elements (Fig. 1A–D). The main cellular population, which constitutes approx 95% of the total gland, is a specialized neurosecretory cell named pineocyte that secretes a number of polypeptides, including melatonin. Pineocytes are modified neurons that have elongated cellular processes with club-shaped terminations that project toward capillary vessels. Their cellular processes can be demonstrated by silver stains similar to neuronal dendrites [5]. Pineocytes have features consistent with their neurosecretory nature, exhibiting clear vesicles, dense-core granules, and synaptic organelles and ribbons [4]. Moreover, pineocytes may express neuronal associated proteins, including neurofilament protein and synaptophysin (Fig. 1C) [6].

The second cell type in the pineal is the stromal-supporting glia that resembles astrocytes of the central nervous system (CNS). These stromal glial cells tend to surround the blood vessels and infiltrate the glandular parenchyma (Fig. 1D). Externally, arachnoidal cells of the leptomeninges encircle the gland. Extracellular calcification, also known as corpora arenacea, is commonly seen and appears to be more prominent with aging.

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Pineocytes are believed to represent modified neurons associated to retinal photoreceptors. In many lower vertebrates, the pineal gland has a direct photoreceptor function that appears to be increasingly reduced and then completely lost during the course of phylogeny [7]. In humans, the pineal gland and the retina share several histogenetic features that reproduce their analogous photosensory ontogeny. Approximately 5–10% of pineocytes express retinal proteins, including S-antigen [7,8] and rhodopsin [7]. However, the role of human pineocytes in photoreception and phototransduction appears to be insignificant.

The pineal gland of all vertebrates, including humans, appears to be able to secrete a number of peptide hormones [3,7,9,10]. Melatonin is the primary hormone of the pineal gland. The major physiological role of melatonin is its influence on circadian rhythmicity to changes in photoperiodism, acting as a photoneurodendocrine transducer and biological pacemaker [10,11]. In addition to melatonin, a variety of peptide hormones are found in the pineal gland and presumably reach the gland by way of pineal connections with the hypothalamus and brain stem. These include peptides such as arginine vasopressin, arginine vasotocin, oxytocin, neurophysins I and II, pineal antigonadotropin, and a gonadotropin-releasing factor unique to the pineal [2,3,9]. The pineal gland appears to interact with various endocrine and neuroendocrine tissues to influence their metabolic activity. The pineal has an inhibitory effect on gonadal function and is believed to affect the timing of puberty [9]. It also appears to play a role in the immunosystem and cancer modulation [12–16]. A wealth of data has been accumulated with respect to the role of melatonin and other pineal hormones on human physiological and pathological states in the last decades. The discussion of these data related to the secretory functions of the normal pineal gland is beyond the scope of this chapter. Further details of such discussions may be obtained from specialized books and review articles.

NON-NEOPLASTIC LESIONS OF THE PINEAL GLAND

PINEAL CYSTS Cysts of the pineal are the most common non-neoplastic lesions of the pineal gland. Small asymptomatic cysts are common incidental finding in neuroradiologic exams performed due to unrelated symptoms, and their incidence may be as high as 10.8% [17]. Cysts are also a frequent finding at autopsies, with an incidence ranging from 25% to 40% [18]. The great majority of pineal cysts are asymptomatic.

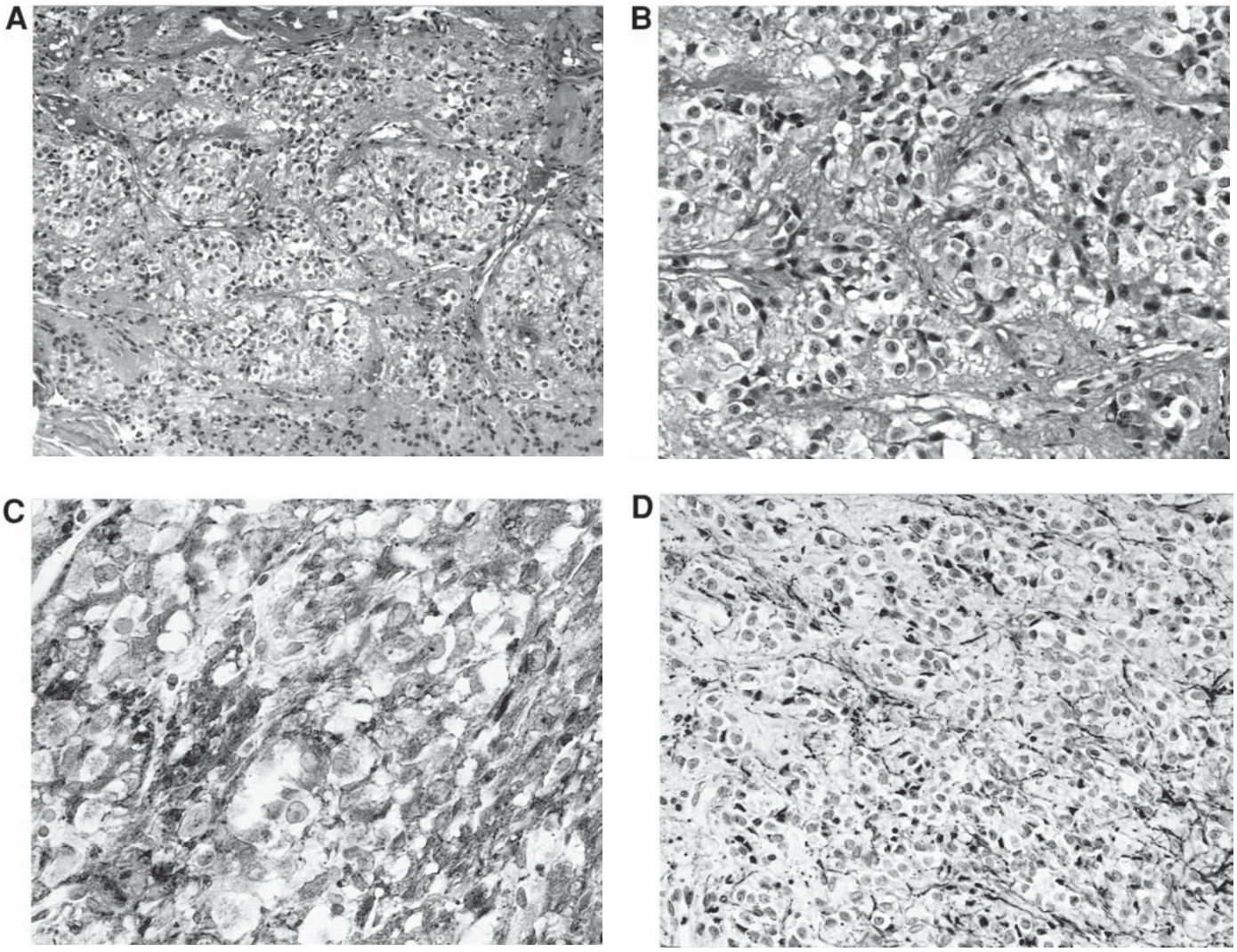

Figure 1 Normal pineal gland. (**A**) The normal pineal gland has a distinct lobular architecture with a prominent fibrovascular stroma. (**B**) The pineocytes are regularly arranged in the lobules displaying prominent cytoplasm and the typical neuroendocrine salt-and-pepper chromatin distribution. (**C,D**) Pineocytes are immunoreactive for neuroendocrine markers including synaptophysin (C), while stromal astrocytes are GFAP reactive (**D**). **A,B**: H&E. **A**: ×100, **B**: ×200; **C**: ABC immunoperoxidase for synaptophysin: ×400; **D**: ABC immunoperoxidase for GFAP: ×200; original magnifications. (**C** and **D** appear in color in the insert following p. 148.)

However, cysts 1.0 cm or larger may cause neurologic symptoms with signs of increased intracranial pressure due to aqueductal obstruction and tectal compression [19–21].

Pineal cysts are glial cysts by nature. The cyst walls are composed of an inner layer of hypocellular glial tissue with dense fibrillary stroma (Fig. 2). Occasional formation of Rosenthal fibers may be seen. The pineal parenchyma surrounding the cyst may demonstrate a variable degree of distortion due to distention of the cyst. Recent and old hemorrhages are commonly observed.

The recognition of pineal cysts is critical, as these lesions may be misdiagnosed as pineal tumors that require additional therapeutic intervention [20].

TUMORS OF THE PINEAL REGION

Tumors of the pineal region (Table 1) represent approx 1% of all brain tumors [22,23], although they account for 3–10% of pediatric brain tumors [24,25]. Moreover, the real incidence of these tumors shows a large discrepancy in different geographic regions of the world. In Japan, their incidence may be as high as 20% owing to the elevated incidence of germ cell tumors arising in this region [26].

The multiplicity of tumors that may arise in the pineal gland is a reflection of the various cell types present in the developing and mature pineal gland. Tumors arising from the pineocytes are the most common and are designated as pineal parenchymal tumors (PPTs). Gliomas, and more frequently astrocytomas, may arise from the supporting glial cells in a smaller proportion of cases. Meningiomas and nonmeningothelial tumors may originate from the meningeal coverings of the pineal gland. However, these tumors may also be secondary invasion of meningiomas originating in the falx or tentorium. The last group of tumors involving this region, accounting for almost 50% of pineal malignancies, are the germ cell tumors [23,25,27,28].

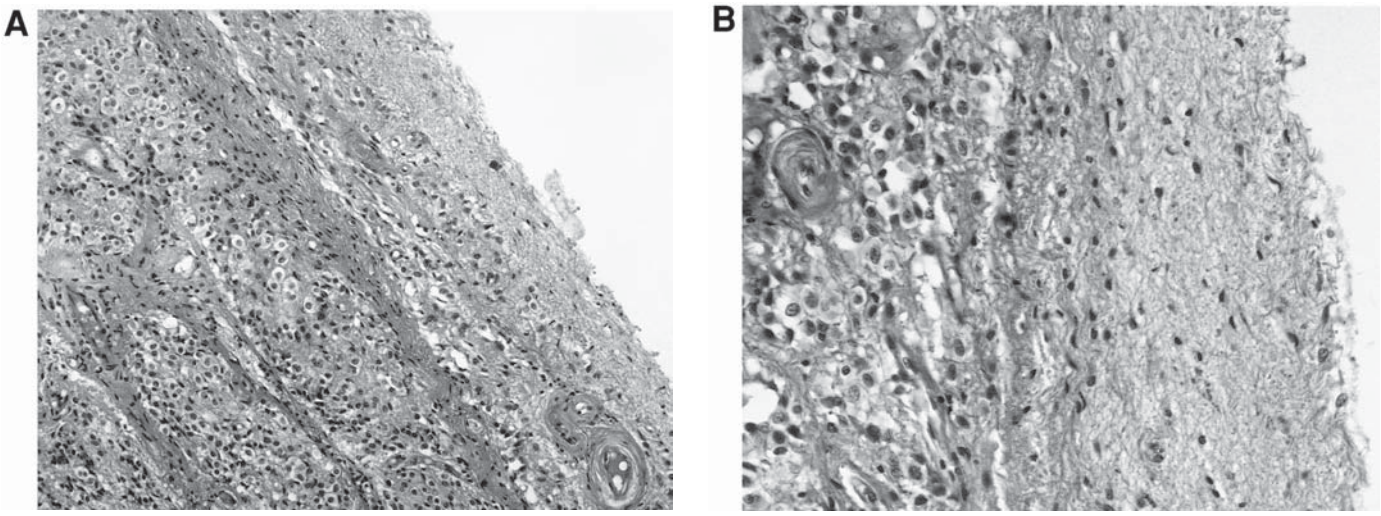

Figure 2 Pineal cyst. (**A**) Pineal cysts showing a hypocellular glial inner layer (*right*) compressing the normal pineal parenchyma (*left*). (**B**) A high-power magnification highlights the glial layer of a pineal cyst. **A,B**: H&E. **A**: ×100; **B**: ×400, original magnifications.

Table 1
Tumors of the Pineal Gland Region

Pineal parenchymal tumors—30%
Pineocytoma
Pineoblastoma
Pineal parenchymal tumor of intermediate differentiation
Primary CNS germ cell tumors—50%
Germinoma
Embryonal carcinoma
Endodermal sinus tumor (yolk sac tumor)
Choriocarcinoma
Teratoma (mature, immature, teratoma with malignant transformation)
Gliomas—15–25%
Astrocytomas
Diffuse infiltrating (WHO grades II–IV)
Pilocytic (WHO grade I)
Ependymomas
Tumors of meninges—5–10%
Meningiomas
Hemangiopericytomas
Miscellaneous tumors
Lymphomas
Lipomas

Table 2
Pineal Parenchymal Tumors

Pineocytoma
Approx 30% of PPT
Arising mostly in adults (median age 35–47)
Grossly well circumscribed tumor
Noninvasive of surrounding brain structures
Histologically well differentiated with characteristic pineocytomatous rosettes
WHO grade II neoplasm
Pineoblastoma
Approx 50–75% of PPT
Commonly arising in the first two decades of life
Slight male predominance
Poorly demarcated tumors with undefined margins with surrounding structures
High tendency to disseminate by CSF pathways
Histologically poorly differentiated, primitive neuroepihtelial cells with high mitotic activity
WHO grade IV neoplasm
Pineal parenchymal tumor of intermediate differentiation
Intermediate grade of PPT with less distinctive clinical and histological features
Includes tumors with either histologic features intermediate between the two previous tumors or tumors that have mixed areas of both tumors
May metastasize by CSF pathways
WHO grade III neoplasm

PINEAL PARENCHYMAL TUMORS

General Considerations PPTs (Table 2) comprise approx-15–30% of the pineal region tumors [27,29–35]. These intrinsic pineal tumors are classified by the World Health Organization Classification (WHO) of Tumors of the Nervous System [33] into three subtypes—pineoblastoma, pineocytoma, and PPT of intermediate differentiation. These three types represent a continuous spectrum of tumoral features ranging from the primitive pineoblastoma to the relatively well-differentiated pineocytoma. Between these two extremes, there are tumors in which differentiation is intermediate that display both pineoblastic and pineocytomatous features.

The three groups of PPTs are also distinctive from the clinical point of view. The primitive pineoblastoma has a clinical behavior similar to other primitive neuroectodermal tumors of the CNS, in which they generally occur in children and young adults, are highly infiltrative tumors, and have the potential for dissemination by the cerebrospinal fluid (CSF) pathways. In general, these tumors behave as a grade IV malignancy. Patients with pineoblastomas have short overall survival time (16 mo in a large cohort study) [34], and low 5-yr progression-free survival (38% in another series) [36]. In contrast, pineocytomas commonly arise in older patients and tend to be more circum-

scribed lesions with no propensity to CSF seeding. Pineocytomas are considered grade II neoplasms by the WHO [33], and survival times are longer than for patients with pineoblastomas (5-yr survival of 67%) [31]. The intermediate PPT appears to have transitional behavior according to the presence or absence of a major pineoblastomatous element [31,33]. These tumors are designated as grade III by the WHO classification [33]. Their potential for local infiltration and CSF dissemination is significant, and their clinical behavior appears to be more aggressive than that of pineocytomas [31].

Histological Types

Pineoblastomas Pineoblastomas are the most primitive of the PPT (Fig. 3). These tumors tend to be more common than the pineocytomas, accounting for up to 18% of the pineal region tumors [27,32,37,38] and for 50–75% of the PPTs [31–33]. They most commonly occur in the first two decades of life [31–33], with a slight male predominance [5,33]. Neuroimaging studies demonstrate multilobulated masses with heterogeneous contrast enhancement and poorly defined margins with the adjacent structures [39]. Pineoblastomas are highly invasive tumors that tend to disseminate through the CFS pathways (Fig. 3D). Extraneural metastases after surgery have been described in two cases: one in a thoracic vertebra and the other in the sacrum [40].

The microscopic findings of pineoblastomas resemble those seen in primitive neuroectodermal tumors of the CNS. They are highly cellular neoplasms composed of small, primitive cells containing round to oval hyperchromatic nuclei and scant cytoplasm (Fig. 3A–C). The cells are arranged in patternless sheets; however, neuroblastic Homer–Wright rosettes may be present. Increased mitotic activity is frequently present (Fig. 3B), and areas of necrosis are common. Endothelial proliferation is present in great number of the cases [35].

Pineoblastomas may show a degree of photosensory differentiation with the formation of occasional photosensory Flexner–Wintersteiner rosettes [5,41,42]. These tumors may also rarely exhibit differentiation to mesenchymal tissues, including striated muscle and cartilage, and melanotic pigmentation [43,44]. Despite the primitive nature of pineoblastomas, a variable degree of neurosensory differentiation is suggested at the ultrastructural level with the findings of bulbous cell processes and 9+0 neurosensory cilia [45–47]. The immunophenotype of pineoblastomas is consistent with both neuronal and neurosecretory differentiation (Fig. 3E) (*see* below). Pineoblastomas are classified as grade IV tumors by the WHO classification [33].

Pineocytomas Pineocytomas account for approx 7–30% of the PPT (Fig. 4). The tumors may occur at any age, but commonly arise in young adults (median age of 35–47 yr) with equal gender distribution [23,31,33,35,48]. In contrast to pineoblastomas, pineocytomas are well-delineated, slightly lobulated tumors with clear margins with adjacent brain structures by neuroimaging [39], consistent with their small tendency of invasion.

Microscopically, they are moderately cellular tumors composed of cytologically mature cells that are arranged in variable lobular formations resembling the normal pineal gland architecture (Fig. 4A,B). Frequently, the cells are arranged in large pineocytomatous rosettes [49] (Fig. 4B). These rosettes resemble Homer–Wright rosettes but on a larger scale, and are characterized by large anuclear, delicately fibrillated areas surrounded by tumor cells. The presence of pineocytomatous rosettes appears to be associated with a more benign clinical course [5]. Papillary arrangements of the tumor cells may be present. Occasional bizarre giant cells can be seen intermixed with the more ordinary cells. This pattern has been recognized by some as "pleomorphic pineocytoma" [35]. However, the presence of pleomorphic cells does not have any prognostic significance [35]. Mitotic figures are rare. Focal necrosis may occasionally be seen.

The neuronal and secretory nature of pineocytomas is demonstrated by immunohistochemical studies (*see* below). At the ultrastructural level, pineocytomas exhibit a relatively mature pineocytic differentiation. Similar to normal pineocytes, the tumor cells show neurosecretory differentiation with cell processes containing microtubules, dense- and clear-core vesicles, and occasional synapselike structures [45]. Neurosensory cilia (9+0) and centrioles are also present [46,47,50–53].

Pineal Parenchymal Tumor of Intermediate Differentiation A significant number of PPTs do not fit accurately into the categories of pineoblastoma and pineocytoma and may exhibit features intermediate between these two distinct tumors. In a recent series of cases classified according to the new WHO guidelines [35], PPTs of intermediate differentiation accounted for 56% of the cases. This category includes tumors with histologic features intermediate between pineocytomas and pineoblastoma or tumors that have mixed areas of both pineocytoma and pineoblastoma. The majority of the tumors show moderate cellularity, mild nuclear atypia, and rare or poorly formed pineocytomatous rosettes. Moderate mitotic activity and areas of necrosis may also be present.

Neurosecretory Differentiation of PPT Immunohistochemical studies have confirmed the neurosecretory differentiation of PPTs. Primarily, these tumors express neuronal-associated proteins including neurofilament proteins, synaptophysin, neuronal-associated class III β-tubulin, microtubule-associated τ and α-synuclein [35,54–57] (Fig. 3E) in variable levels depending of the degree of tumor differentiation. Whereas pineocytomas show intense reactivity for the majority of these markers, pineoblastomas may show reactivity to a few markers and with lower intensity [35]. Similarly, neuroendocrine markers such as chromogranin and neuron-specific enolase (NSE) are seen in the majority of the tumor but in proportion to the tumor degree of differentiation [35]. Glial fibrillary acidic protein (GFAP) and S100 protein may be seen in the interstitial glial cells of the tumors, but are not present in tumor cells.

In addition, these tumors may also express photoreceptor-associated proteins reflecting the transient photosensory differentiation of pineocytes during development [7]. The photosensory-associated protein retinal S-antigen (S-Ag), a photoreceptor membrane-associated soluble protein, has been reported in pineoblastomas [58], pineocytomas [35,58–60], and intermediate PPTs [35]. Another photosensory marker, interphotoreceptor retinoid-binding protein (IRBP), an interphotoreceptor matrix protein that functions in retinoid transport between the photoreceptor cells and the retinal pigment epithelium, has also been demonstrated in a PPT of intermediate differentiation [55].

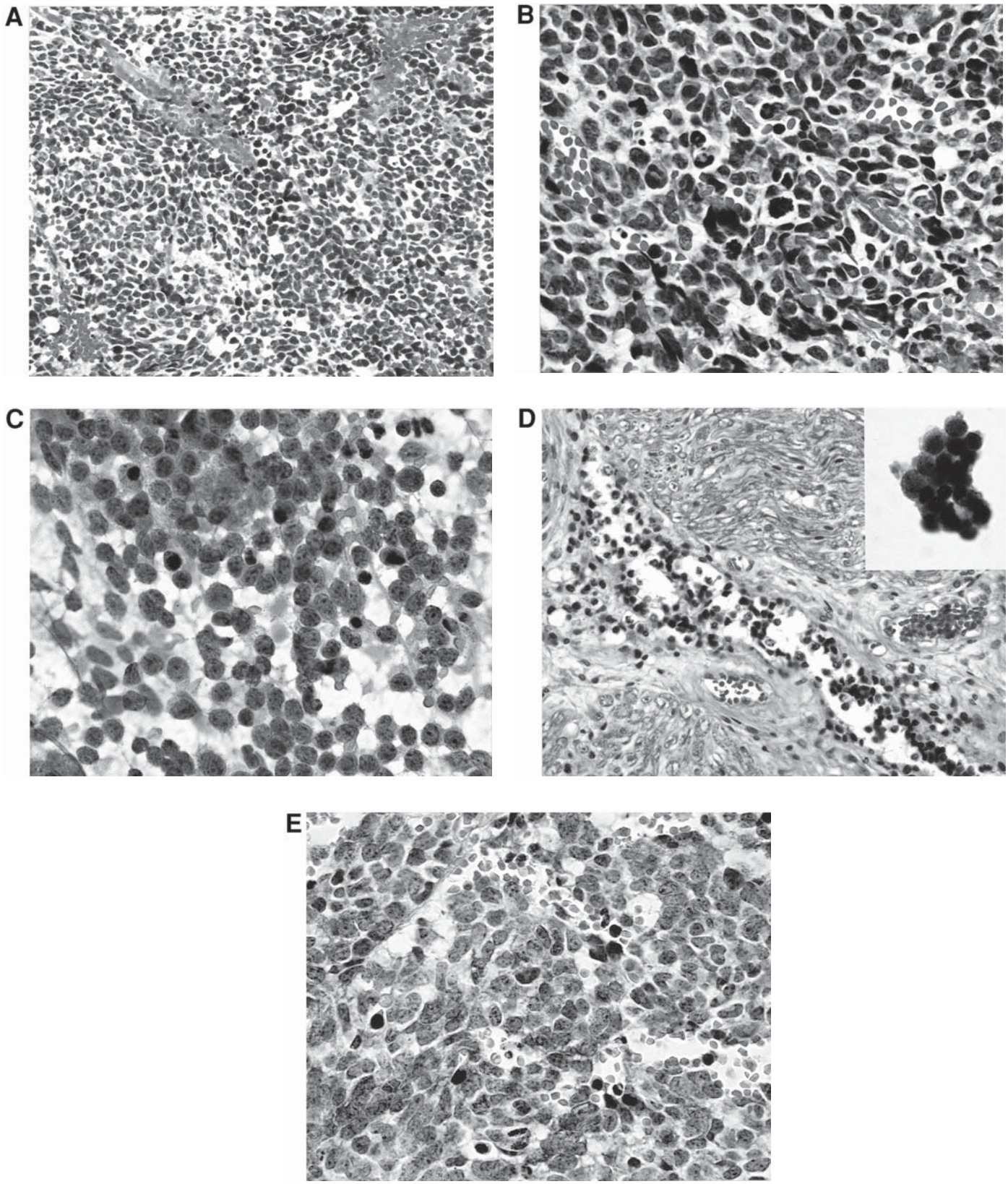

Figure 3 Pineoblastoma. (**A**) Pineoblatomas are hypercellular tumors composed of small, undifferentiated cells with hyperchromatic nuclei. (**B**) Mitotic figures may be conspicuous (*center*), and foci of necrosis are commonly present. (**C**) Smear preparation of a pineoblastoma accentuates the hyperchromatic features of the cells. (**D**) Pineoblastomas are prone for dissemination via the CSF pathways. In this example, CSF was positive for tumor cells (*inset*) and the autopsy revealed involvement of nerve roots by the tumor. (**E**) Synaptophysin is demonstrated in pineoblastoma. **A–D**: H&E. **A**: ×100; **B**: ×400; **C**: ×400; **D**: ×200; **D** *inset*: Papanicolau, ×400; **E**: ABC immunoperoxidase for synaptophysin, ×400; original magnifications. (**B** and **E** appear in color in the insert following p. 148.)

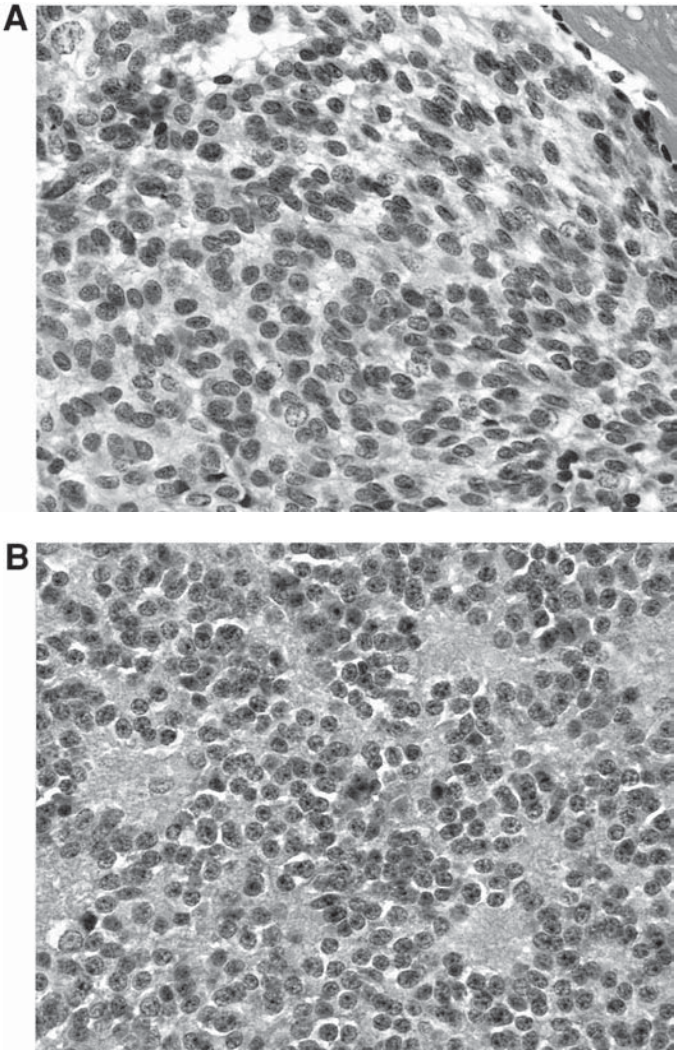

Figure 4 Pineocytoma. (**A,B**) Pineocytomas are composed of a uniform population of differentiated, "mature" cells arranged in a solid sheet (**A**) and/or in the characteristic "pineocytomatous" rosette (**B**). Pineocytoma displaying elongate, delicate neuritic processes within the center of the rosettes. **A,B**: H&E, ×200, original magnification.

Markers of Prognostic Value Very few studies have evaluated morphological features of PPTs as prognostic and/or predictive factors. A recent series of 66 cases of PPT [35] analyzed a number of histological and immunohistochemical features and their role in patient survival and tumor recurrence. These authors have found that the number of mitoses (>6) and the presence of necrosis were associated with shorter disease-free and overall survival. On the other hand, neuronal differentiation of the tumor, as evident by neurofilament immunolabeling, was significantly associated with a higher survival rate.

Molecular and Cytogenetic Profile of PPTs The mechanisms involved in the tumorigenesis of PPT remain incompletely understood. Molecular and cytogenetic data on PPT are sparse, probably owing to the rarity of these tumors. Cytogenetic studies performed on single cases of pineocytomas and pineoblastomas have found few distinctive chromosomal abnormalities in these tumors. Pineocytomas have shown losses of chromosomes 11 and 22 and deletions in the distal 12q region, while pineoblastomas exhibited deletion of chromosome 11q [61–64]. Comparative genomic hybridization (CGH) has been performed recently in a series of nine PPTs [65]. Although no chromosomal gains or losses were found in pineocytomas (three cases), the most frequent DNA copy number changes in pineoblastomas (three cases), and PPTs of intermediate differentiation (three cases) were gains of 12q, 4q, 5p, and 5q, and losses of 22, 9q, and 16q. Nevertheless, the number of tumors that have been analyzed so far is too small to permit any firm conclusions on the role of these chromosomal abnormalities in the pathogenesis of these tumors.

Analysis of the *p53* tumor suppressor gene in PPT revealed neither *p53* gene mutations nor overexpression of p53 protein by immunohistochemistry, suggesting that the *p53* gene pathway may not be involved in the tumorigenesis of these tumors [66].

TUMORS OF THE NEUROGLIA Gliomas involving the pineal region are less common than the PPTs, with an incidence ranging from 15% to 25% of tumors of the region [32,34]. Astrocytomas are the most common gliomas involving the pineal and are hypothesized to derive from the supporting glial of the normal pineal gland. Other gliomas may also involve the pineal region, but not necessarily arise from the pineal gland itself. Astrocytomas of the thalamus, brain stem, or corpus callosum commonly infiltrate the quadrigeminal plate cistern and may appear as a pineal region mass. Ependymomas and choroid plexus papillomas are thought to involve the pineal as a result of the close proximity of the gland to both ependyma and choroid plexus of the posterior roof of the third ventricle [67]. In a European cohort of 281 pineal region tumors, gliomas represented 25% of the cases, and these include astrocytomas (52/72), ependymomas (13/72), and oligodendrogliomas and mixed oligoastrocytomas (7/72) [34].

All variety of astrocytomas have been reported as involving the pineal, including the well-delineated and relatively benign pilocytic astrocytoma (WHO grade I) and the diffuse infiltrating astrocytomas (grades II–IV) [27,68–72] (Fig. 5). Pilocytic astrocytomas of the pineal region behave in a similar benign fashion as the ones arising in other locations of the CNS. Owing to the circumscription of the tumors, they frequently are totally resected, resulting in a favorable prognosis of the patients [27, 32]. Diffuse infiltrating astrocytomas are associated with a poorer prognosis. Most of the cases preclude total surgical resection, with management of the tumor by diagnostic biopsy only followed by radiotherapy [32]. In addition, diffuse astrocytomas may affect the subjacent brain parenchyma. Although all grades of astrocytomas have been reported as involving the pineal gland, high-grade astrocytomas (anaplastic astrocytoma and glioblastoma) are less common at this localization than the low-grade astrocytomas [27,32,71,72].

GERM CELL TUMORS

General Considerations Germ cell tumors (GCTs) comprise the largest group of neoplasms in the pineal region, representing nearly 50% of the tumors involving the region [23,25, 27,28,32,34]. Nevertheless, their incidence varies according to geographic area, with the highest numbers of cases occurring in Asia. Most of the tumors arise in the first two decades of life, with the majority of the patients diagnosed between 11 and 20 yr of age [23,32,33,73]. A sex predilection is seen regardless of

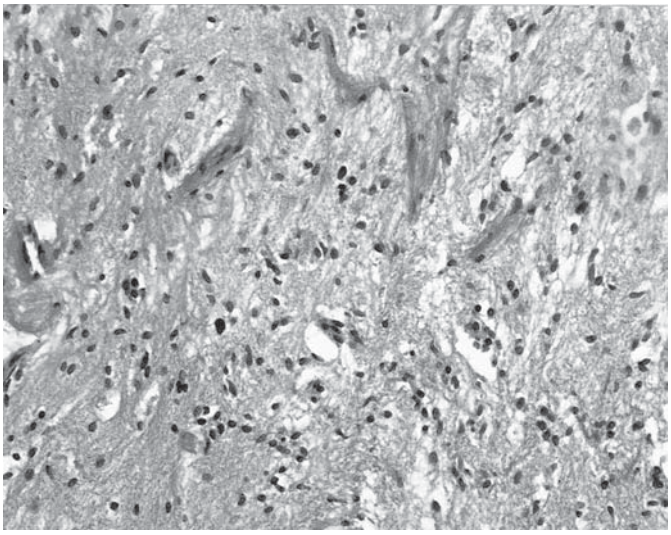

Figure 5 Gliomas. An example of diffuse astrocytoma (WHO grade II) of the pineal region showing low-grade features, similar to astrocytomas of other regions of the CNS. H&E, ×200.

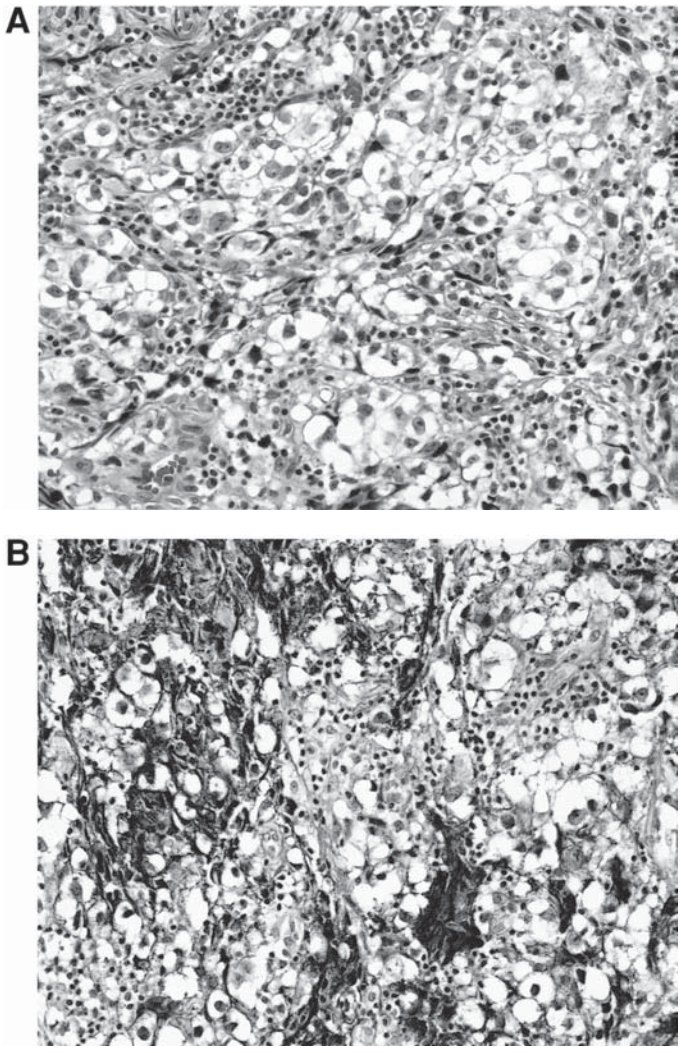

Figure 6 Germinoma. (**A**) Germinomas are composed by dual populations of large, pleomorphic polygonal cells and small lymphocytes infiltrating the fibrovascular stroma. (**B**) The large tumor cells are distinctly positive for placental alkaline phosphatase (PLAP). **A**: H&E, ×200; **B**: ABC immunoperoxidase for PLAP, ×200; original magnifications.

the type of GCT, with a male-to-female ratio of 11:1 in one series [32]. The association of GCT of the pineal region with GCT in other locations in the brain, particularly the suprasellar region, is not uncommon, ranging from 2% to 12% of the reported cases [74].

The group of GCTs includes five categories: germinoma, embryonal carcinoma, choriocarcinoma, endodermal sinus (yolk sac) tumor, and teratoma [33]. Mixed GCT, that is, tumors with two or more histological types, may also occur in a high percentage [31]. GCTs arising in the pineal region are histologically identical to those occurring in other regions of the CNS, in the gonads, and at other extragonadal sites.

The histogenesis and differentiation of pineal GCT appear to be similar to those of tumors of other gonadal or extragonadal location, and are believed to arise from the neoplastic transformation of primordial germ cells. It is hypothesized that during embryonic development, most of the primordial germ cells migrate to the urogenital ridges, the origin of the gonads. However, primordial cells may also disseminate in an aberrant fashion to other tissues, particularly along midline structures including the mediastinum and thymus, and the CNS at the diencephalopineal region [75]. Nevertheless, the mechanisms involved in malignant transformation of the extragonadal primordial germ cells are still unknown. It has been suggested that neuroendocrine factors may play a role in neoplastic transformation of the cells in the intracranial locations, mainly due to the proximity of the primordial cells to diencephalic centers for gonadotropin regulation. In addition, the clinical presentation of the majority of the germ cell tumors coincides with the changes occurring in this region at the time of puberty [73,74]. For an update discussion on the possible histogenetic theories of the CNS GCTs, the reader should refer to Rosenblum et al. [76].

Histological Types

Germinoma Germinomas are the most frequent GCT involving the pineal region, accounting for 50–80% of such tumors [23–25]. Germinomas of the pineal are typically well-circumscribed solid tumors with an obvious plane between tumor and normal tissues. However, some cases may invade the adjacent brain. Grossly, the tumors are soft and friable masses with variable cystic component. Areas of necrosis and hemorrhage are infrequent.

Germinomas are composed of a uniform population of large polygonal cells with pale to clear cytoplasm due to the presence of abundant glycogen. A large, vesicular nucleus is centrally located within the cell, usually containing one or more prominent nucleoli (Fig. 6). The tumor cells are arranged in large lobules separated by delicate fibrovascular septa. The latter contain lymphoid or lymphoplasmacellular infiltrates with large numbers of T lymphocytes and activated macrophages [77]. A granulomatous reaction with aggregate of epithelioid histiocytes may also be present [78].

Table 3
Immunohistochemistry of Germ Cell Tumors

Tumor	*PLAP*	*AFP*	*β-HCG*	*Cytokeratin*
Germinoma	+	–	–	±
Embryonal carcinoma	+	±	±	+
Endodermal sinus tumor	±	+	–	+
Choriocarcinoma	±	±	+	+

+, Immunoreactive; ±, may be focally immunoreactive; –, immunonegative.

Immunohistochemistry of germinomas shows reactivity for placental alkaline phosphatase (PLAP) [78–80] (Fig. 6B), a useful marker for clinical follow-up of tumor relapses [80]. Staining for PLAP is most commonly seen as a surface membrane pattern and less frequently as diffuse cytoplasmic stain. Human chorionic gonadotropin (β-hCG) may be sporadically positive in typical tumor cells [79]; however, a β-hCG stain may also indicate the presence of syncytiotrophoblastlike cells within the tumor [81]. Germinomas with syncytiotrophoblastic elements appears to behave differently than ordinary germinomas. There are reports of higher recurrence rate [82] and shorter survival time [83] in β-hCG-producing germinomas than pure germinomas. However, as compared to other GCTs, germinomas have a more favorable prognosis.

Embryonal Carcinoma Embryonal carcinomas are very primitive GCTs, second only to germinomas. They are rare as pure tumors, constituting only approx 5% of all intracranial germ cell tumors [73]. More frequently, embryonal carcinomas are a component of mixed GCTs, mostly associated with immature teratomas and choriocarcinomas. Embryonal carcinomas are usually large tumors that have a firm, fibrous consistency. The tumors have a rich vascular supply and tend to encase major blood vessels of their surroundings, which complicates their total surgical resection [25].

Histologically embryonal carcinomas are composed of large polygonal cells that proliferate in cohesive nests and sheets. The cells contain large, vesicular nuclei with prominent nucleoli. The tumor cells may also be arranged in epitheliallike arrangements including papillae and glandlike structures. High mitotic activity and extensive areas of necrosis are commonly present.

Immunohistochemical profile of embryonal carcinomas is remarkable for the diffuse cytokeratin reactivity that distinguishes this tumor from germinomas (*see* Table 3). PLAP is present in the majority of the tumor cells whereas AFP and β-hCG may be seen in a variable number of cases [81,84].

Endodermal Sinus Tumor (Yolk Sac Tumor) Endodermal sinus tumor is a highly malignant GCT. In the majority of the cases survival rates do not exceed 14 mo [85]. These tumors represent approx 7% of intracranial germ cell tumors [32,73], and their preferential location is the pineal region [78,85]. However, approx 50% of the pineal cases are in fact mixed GCTs containing various proportions of endodermal sinus tumor and other germ cell elements [85].

Endodermal sinus tumors are characterized by the presence of pseudoglandular structures formed by endodermal cells arranged in a myxoid matrix [5,79,86]. The tumor cells are often clear, cuboidal to columnar epitheliallike cells arranged in sheets, cords, and variable tubular formations to true papillary structures (Fig. 7A,B). The presence of distinct perivascular epithelial-lined structures, named Shiller–Duval bodies, are typically seen in these tumors. Other common features are the presence of eosinophilic, periodic acid-Schiff (PAS)-positive and diastase-resistant hyaline droplets located in the cytoplasm or free in the stroma [81].

Endodermal sinus tumors are characteristically immunoreactive for α-fetoprotein (AFP) in both the epithelial cellular component (Fig. 7B) as well as the hyaline globules. In fact, elevated AFP in CSF and/or blood alone appears to be strongly suggestive of an endodermal sinus tumor [87].

Choriocarcinoma Pure choriocarcinomas are extremely rare [28,88], but the pineal region appears to be the preferential site for these tumors, accounting for approx 75% of the intracranial cases [5]. More frequently, choriocarcinoma is one of the components of a mixed GCT. Choriocarcinomas are often associated with precocious puberty with elevations of β-hCG and/or luteinizing hormone [73].

The tumor is characterized by extraembryonic differentiation along trophoblastic lines, and microscopically they are composed of syncytiotrophoblasts and cytotrophoblasts arranged in a bilayer structure. Choriocarcinomas are typically hemorrhagic masses with a rich sinusoidal vasculature. As a matter of fact, in mixed GCTs, the hemorrhage may completely obscure foci of choriocarcinomatous differentiation. The syncytiotrophoblasts are the source of β-hCG elevations in serum and CSF. β-hCG can be easily identified within tissue by immunohistochemistry [79,88]. PLAP and cytokeratin are also positive in the majority of the tumors [78,84].

Teratoma Teratomas are tumors composed of a mixture of tissues derived from the three embryonic germinal layers and are, consequently, considered to be neoplastic counterparts of embryonic tissues. These tumors constitute approx 0.5% of all intracranial neoplasms [22], and the pineal region is the most common site, where they represent nearly 15% of the pineal GCTs [5,22,25,31,89]. The tumors usually occur in younger patients than do germinomas, with a median age of 11 yr at presentation [89]. Similar to germinomas, there is a marked male predominance.

Teratomas are often large, well-circumscribed masses that tend to adhere firmly to the adjacent parenchymal structures. The tumors are generally multicystic and composed of a mixture or recognizable mature elements including keratin balls, hair, cartilage, and bone. Immature elements are less obvious, but are commonly associated with areas of necrosis and hemorrhage. The histologic appearance of teratomas varies according to the presence of these immature elements and their degree of differentiation. The two principal variants of teratomas are mature and immature teratomas.

Mature teratomas are relatively less common than the immature teratomas. The majority of tumors are cystic and show diverse gross appearances due to the presence of the different tissues and elements. Histologically, they are composed of fully differentiated, mature tissues representative of the three germinal layers. These are often organized in an ordered pattern resembling adult tissues, for example, skin with adnexa, cartilage and

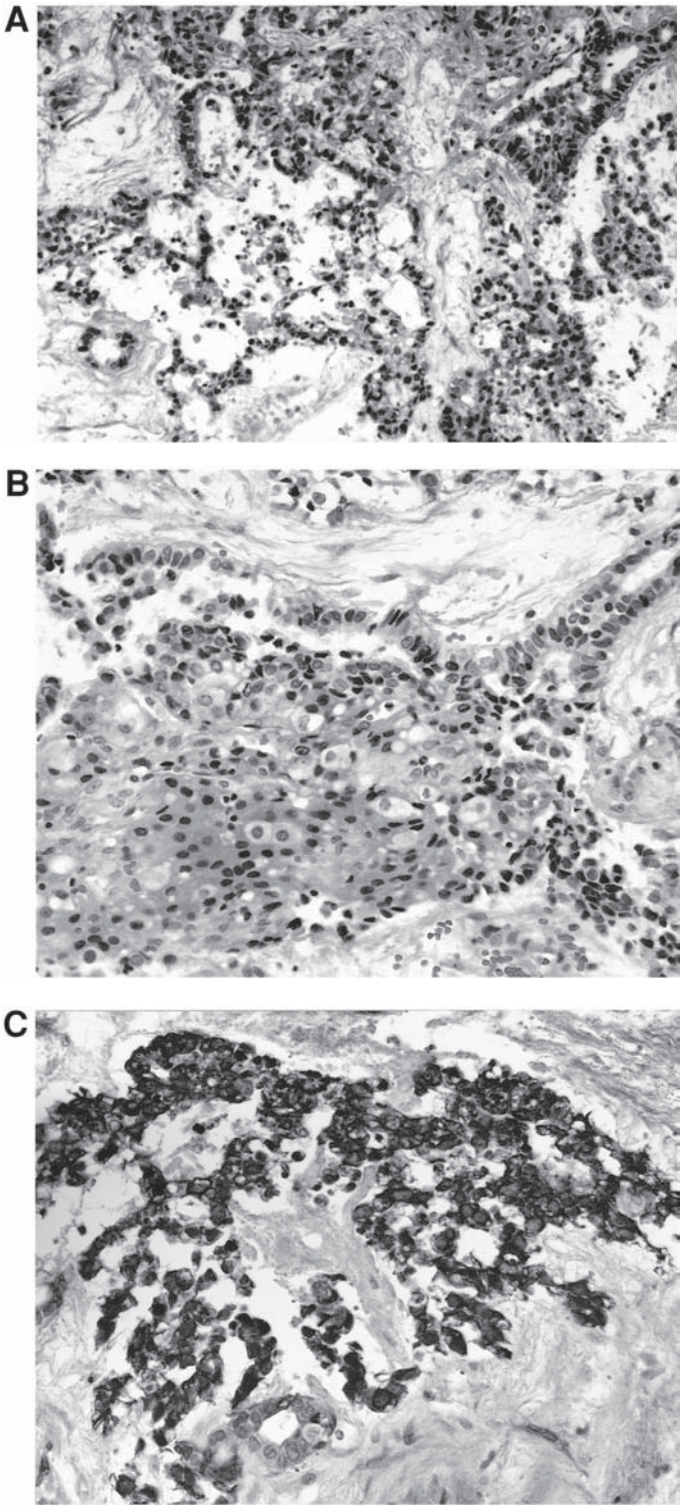

Figure 7 Yolk sac tumor. **A,B**. An example of yolk sac tumor showing papillary structures (**A**) admixed with a more diffuse cellular arrangement (**B**). **C**. α-Fetoprotein is characteristically reactive in these tumors. **A,B**: H&E. **A**; ×100; **B**: ×200; **C**: ABC immunoperoxidase for α-fetoprotein, ×200; original magnifications. (**C** appears in color in the insert following p. 148.)

bone, adipose tissue, bundles of striated and smooth muscle, glioneuronal tissue with choroid plexus, retina with pigmented ocular epithelium, and so forth.

Immature teratomas are the most common form of intracranial teratomas [28,32]. They are composed of incompletely differentiated or immature elements derived from one or more of the three germinal layers. The major component of immature elements is neuroepithelial in nature, including embryonic medullary epithelium, primitive rosettes, or more specialized structures such as Flexner–Wintersteiner and Homer–Wright rosettes similar to those of retinoblastomas and neuroblastomas, respectively. Immature elements derived from other germinal layers are less common. Immature teratomas appear to have a less favorable clinical course compared to mature teratomas [90]. Immature teratomas tend to disseminate to the CSF pathways more frequently [28,91,92], and recurrence and death rates are higher than mature teratomas [28,90]. However, the presence of immature elements does not indicate a malignant transformation of these teratomas (*see* below). Maturation of the immature elements may rarely occur in residual or recurrent tumors [93].

Teratomas with malignant transformation are tumors in which there is an association of a conventional "malignant" component of somatic type, for example, carcinomas and sarcomas, with the regular mature or immature teratomatous elements [30]. Rhabdomyosarcoma or undifferentiated sarcomas are the most commonly seen [33]. These tumors are less frequent than the previous types of teratomas. The designation of "teratoma with adenocarcinoma" or "teratoma with rhabdomyosarcoma" should be used instead of the generic designation of "malignant teratoma."

The association of malignant germ cell elements may often be seen in both mature and immature teratomas, particularly germinoma and embryonal carcinoma [5,33]. The association between germinoma and teratoma has been estimated to be about one fifth of teratomas [94]. In these mixed germ cell tumors, the denomination of "teratoma with germinoma" or "teratoma with an embryonal carcinoma" appears to be more adequate than the old expression "teratocarcinoma."

Cytogenetics and Molecular Genetics Cytogenetical analyses of intracranial GCT have occasionally been reported, but no definitive cytogenetic profile has emerged from these studies. Numerical or complex structural chromosomal abnormalities were seen in the majority of the cases analyzed [95–99]. The presence of isochromosome 12p [i(12p)], the most common karyotypic abnormality of gonadal GCT [100], has been demonstrated in several cases [97,99–102], suggesting analogous genetic pathways in the formation of gonadal and intracranial GCT.

Prognosis and Predictive Factors The histological type of GCT seems to be the most important prognostic factor for a patient's survival. Comparatively, pure germinomas have longer survival and lowest recurrence rates than any other GCTs [82, 83]. The 5-yr survival rate for pure germinomas ranges from 90% to 95% while 10-yr survival may reach 91% [82,83]. Malignant GCTs including choriocarcinoma, embryonal carcinoma, yolk sac tumor, and mixed germ cell tumor have the lowest survival rates, with 5-yr survival of approx 44% [31,83]. Survival rates associated with teratomas vary according to their degree of differentiation. While mature teratomas have 5-yr survival rates as high as 93%, immature teratomas and teratomas

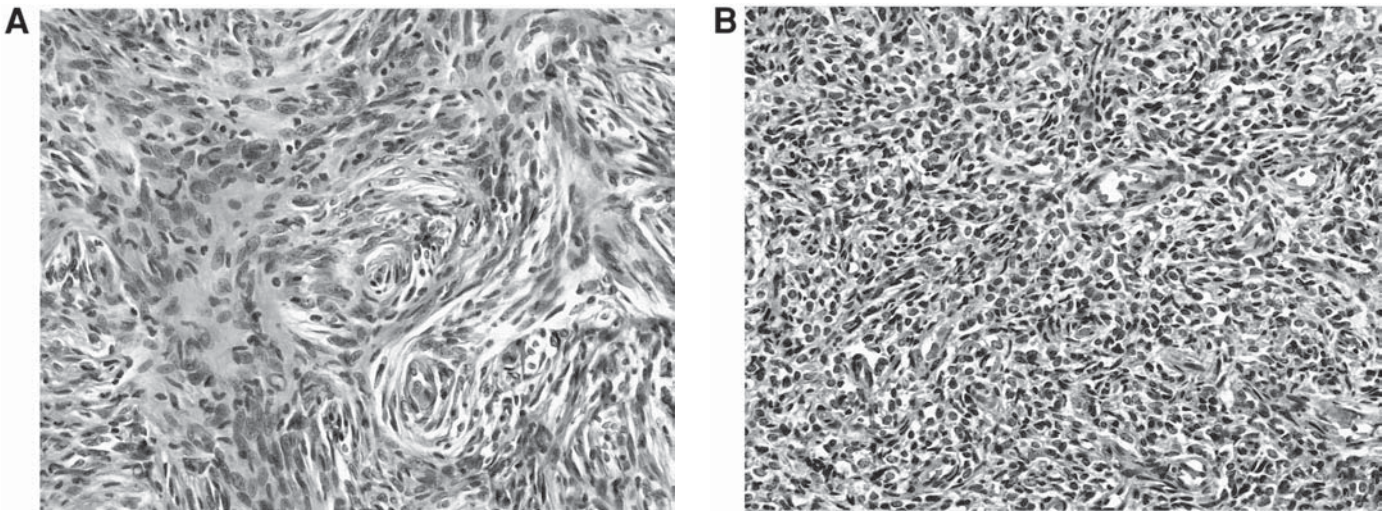

Figure 8 Meningeal tumors. (**A**) A meningothelial meningioma arising in the pineal gland of a 36-yr-old female. (**B**) An example of hemangiopericytoma involving the pineal region. **A,B**: H&E. **A**: ×200; **B**: ×100; original magnifications.

with malignant transformation have a lower 5-yr survival rate of 75% [83].

TUMORS OF MESENCHYMAL ORIGIN

MENINGIOMA AND NONMENINGOTHELIAL TUMORS OF THE MENINGES Meningiomas of the pineal region are rare and comprise approx 5–10% of tumors of this region [103–106]. Pineal region meningiomas may involve the pineal region in two manners. They are believed to arise from the velum interpositum, a double fold of pia–arachnoid that forms the roof of the third ventricle and surrounds the pineal gland [107]. Alternatively, meningiomas may arise from the junction of the falx and tentorium and secondarily invade the posterior third ventricle and consequently the pineal gland.

Pineal meningiomas appear to occur in a younger population than tumors in other locations [105,107]. In the three largest series of meningiomas arising in the pineal region [105,106, 108] the mean ages were 28, 41, and 49 yr and lower than that reported for meningiomas in general (peak 50–59) [33]. Similar to meningiomas of other CNS regions, there is a female predominance. All histological subtypes of meningiomas have been described in the pineal region [107] (Fig. 8A).

Other mesenchymal tumors involving the meninges have been reported to occur at this location including hemangiopericytoma [109,110] (Fig. 8B) and lipomas [92].

MISCELLANEOUS TUMORS The pineal gland may rarely harbor metastatic carcinomas. There have been few reports of lymphoma involving the pineal gland [111, for review] and a single case of primary Langerhans' cell histiocytosis [112].

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7 The Parathyroid

H. RUBÉN HARACH, MD, FRCPATH

HISTORICAL BACKGROUND

The human parathyroids were recognized, histologically described, and named "glandulae parathyreoideae" by Sandström in 1880. The elucidation of hyperparathyroidism was principally made by the contributions of the Austro–German pathological school and the American physiological school. Gley was the first to demonstrate in 1891 the functional importance of the parathyroid glands by showing that their removal accounted for the fatal seizures in dogs following thyroidectomy. In 1891 Von Recklinghausen described the pathologic bone changes of osteitis fibrosa cystica; its association with a tumor of possible parathyroid origin was made in 1904 by Askanazy. The connection of parathyroid hyperplasia with bone disease was also suggested by Erdheim in 1903, but he considered that parathyroid gland abnormalities occurred secondarily to the bone disease, correctly as we now know for osteomalacia, but not for parathyroid adenomas. Still in 1903, Erdheim deserves the credit for first describing an (multiple endocrine neoplasia [MEN1]) acromegalic patient showing a pituitary tumor and enlarged parathyroid glands at autopsy. In 1915 Schlagenhaufer proposed that the bone disease was the result rather than the cause of the parathyroid tumors. In 1925 in Vienna Mandl removed for the first time a parathyroid tumor (probably a carcinoma) from a patient with osteitis fibrosa cystica who had a dramatic relief of the bone disease. The connection between parathyroids, calcium, and tetany was made by MacCallum and Voegtlin in 1908, with the isolation of parathyroid hormone (PTH) being accomplished independently by Hanson in 1924 and Collip in 1925. A tentative diagnosis of overproduction of PTH (hyperparathyroidism) was made for the first time in 1926 by DuBois, who was initially in care of an American merchant seaman—Captain Charles Martell. He had osteitis fibrosa cystica and his calcium metabolism was reported to be similar to that of a man receiving 100 U of parathyroid extract a day as described by Aub and Bauer in 1930. The seaman underwent six surgical neck explorations without success, and, on his seventh exploration in 1932, a parathyroid tumor was found in his anterior mediastinum. The renal and homeostatic effects of PTH were studied by several investigators, but probably Albright was the most outstanding in this new era, beginning with a paper read in 1923 as a fourth-year medical student. Within the pathological spectrum of parathyroid disease he recognized water-clear cell hyperplasia in 1934 and, in 1941, suggested that hypercalcemia and hypophosphatemia found in a patient with a metastatic hypernephroma might be due to a PTH-like substance (now known to be parathyroid hormone-related peptide [PTHrP]) secreted by the tumor. In 1958 Cope and collaborators recognized another type of primary chief cell hyperplasia [1–4]. This historical approach merges into that of our times with the contribution of several investigators to the elucidation of the physiological, biological, molecular, and genetic mechanisms involving the parathyroid glands in normal and pathological conditions [5]. In practice, the management of parathyroid disease, especially hyperparathyroidism, requires a multidisciplinary approach that includes the participation of the endocrinologist, surgeon, and pathologist. This chapter deals mainly with the morphological aspects of parathyroid disease and differential diagnoses from the aspects of pathology. The molecular aspects of the parathyroid glands are thoroughly considered in another chapter.

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THE NORMAL PARATHYROID

EMBRYOLOGY AND ANATOMY The parathyroid glands derive from the endodermal third and fourth branchial pouches, which connect with the pharynx by the ductus pharyngobrachialis. The lateral and ventral walls of the third and fourth pouches are replaced by parathyroid cells, which are first recognized in 5–6 wk human embryos. The third pharyngeal pouch wall extends caudally beyond parathyroid III (inferior) and forms thymus III from its ventral part (Fig. 1). In the-6-wk embryo the parathyroid III and thymus III complex is completely separated from the pharynx. Then thymus III migrates caudally with the developing heart dragging down parathyroid III, which, after separating from thymus III, will finally reach its permanent position at the lower aspect of the thyroid lobe in the 7–8 wk embryo. If this separation fails, the parathyroid III will migrate to the lower neck within the thymus or in to the mediastinum. In contrast, early separation of these two components may result in a more cephalic location of parathyroid III close to the upper pole of the thyroid or superior parathyroid. Similarly, parathyroid IV (superior) develops from the fourth pharyngeal pouch

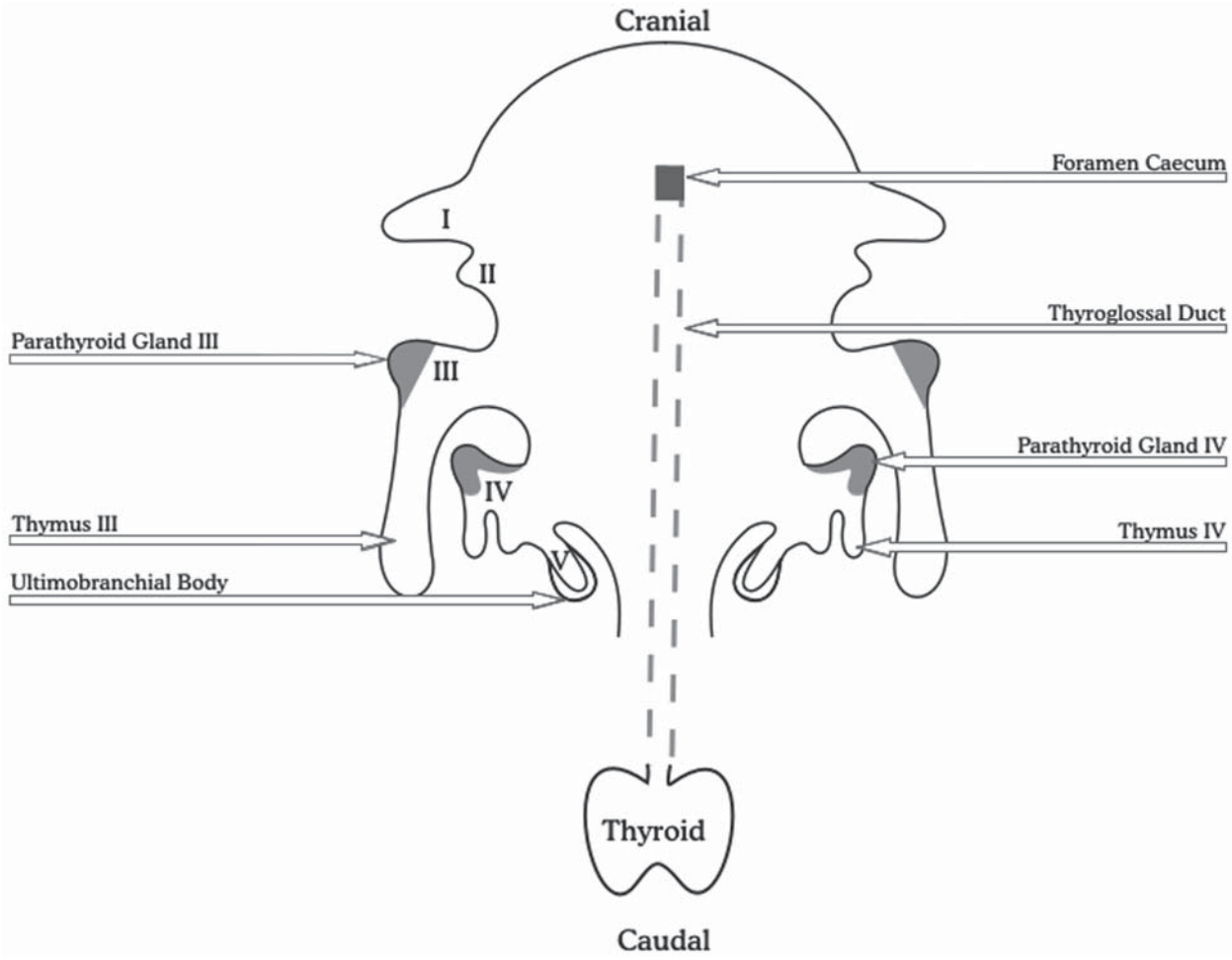

Figure 1 Schematic embryological derivation of the parathyroid glands and thymus.

together with thymus IV (accessory thymus) and the ultimobranchial body (Fig. 1). The parathyroid IV reaches the final position at the upper third of the thyroid lobe in the 18-mm embryo, when the ultimobranchial body starts encroaching on the lateral thyroid lobe and thymus IV tissue locates outside the thyroid [6,7]. The fetal parathyroid is composed of nests of finely vacuolated chief cells with round small hyperchromatic eccentric nuclei and start to show PTH-immunoreactivity from 10 wk of gestation [8]. Oxyphil and fat cells are not present. Classic adult-type chief cells start to manifest in early childhood [8–10].

Most humans have four parathyroid glands. They are tan to red–brown and flattened, oval, or bean-shaped measuring about 6 mm in length by 3–4 mm in width by 1–2 mm in thickness. Occasionally the parathyroids show an elongated or lobulated configuration [11–13]. The combined weight at 6 mo of age is less than 10 mg, at 1 yr 20 mg, at 5 yr 30–40 mg, at 10 years is up to 60 mg, and in adults is approx 140–160 mg. The upper limit weight of individual glands is generally considered as 40 mg [12,14–17]. In about 80% of the cases the superior and inferior pair of parathyroid glands show a symmetrical anatomical position. The superior parathyroids are usually located in connective tissue at the cricothyroid junction between the posterior edge of the thyroid and the pharynx, about 1 cm above the intersection of the recurrent laryngeal nerve and the inferior thyroid artery. Sometimes the glands are found behind the upper pole of the thyroid and very rarely lie in a retropharyngeal or retroesophageal location. The inferior parathyroids are more variable in distribution probably due to their more complex embryological migration pathway. Most inferior parathyroid glands lie lateroposteriorly or ventrolaterally to the lower pole of the thyroid lobe [12,13] (Fig. 2).

HISTOLOGY AND ULTRASTRUCTURE The parathyroid glands are demarcated by a thin connective tissue capsule that may be absent, leaving parathyroid epithelial cell nests admixed with fatty tissue. Failure of removal of such nests in operations for primary or tertiary hyperparathyroidism may lead to persistent hypercalcemia or recurrent hyperparathyroidism. The main epithelial component of the parathyroid glands is the chief cell, which measures approx 12 μm in diameter, and has polygonal clear and finely granular cytoplasm with a 7 μm central round hyperchromatic nucleus with a small nucleolus (Fig. 3). Chief cells contain intracytoplasmic periodic acid-Schiff (PAS)-positive glycogen [9] and oil red O positive or sudanophilic droplets of neutral lipid [18,19], which can also be recognized ultrastructurally together with scattered argyrophil membrane-bound secretory granules measuring approx 150 nm [20–22] (Fig. 4). A decrease in intracytoplasmic lipid is seen in a proportion of

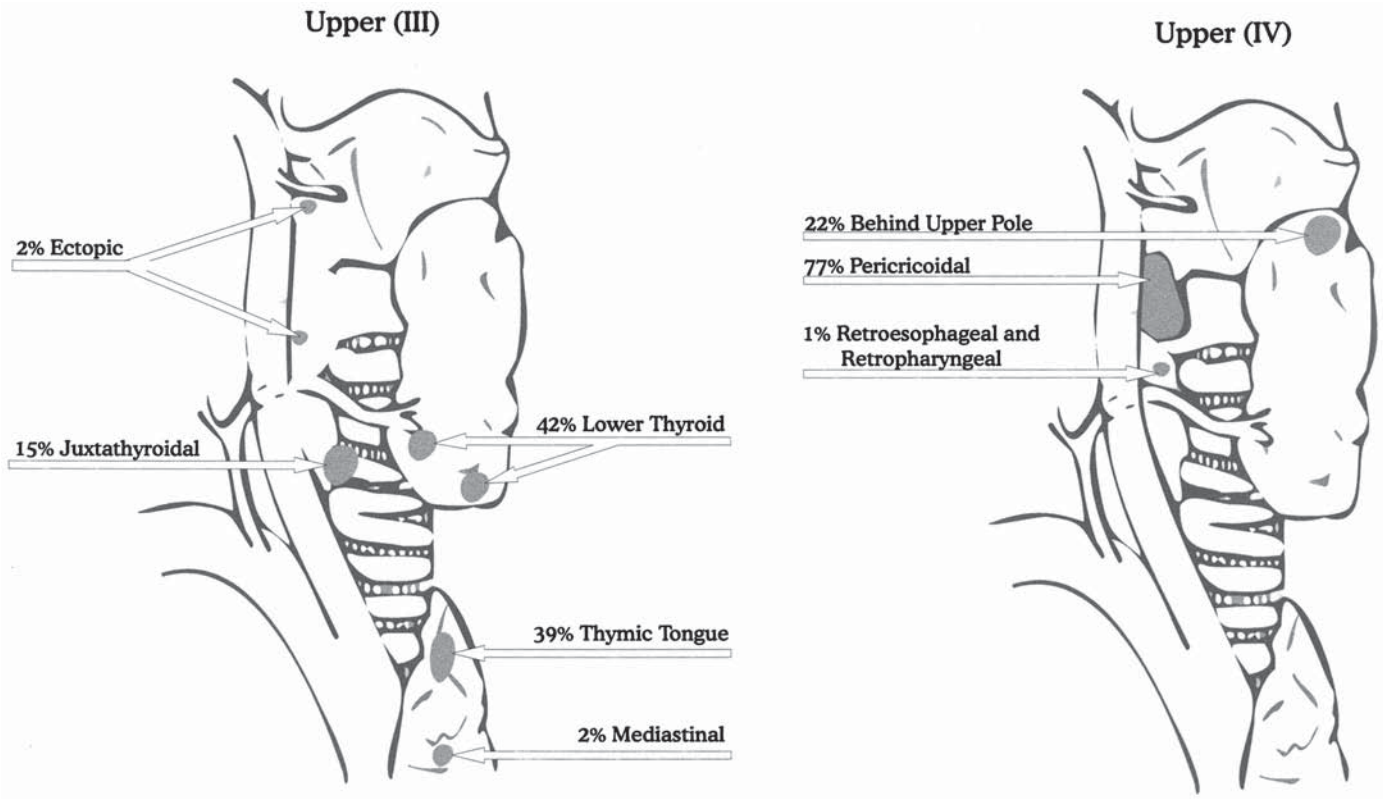

Figure 2 Anatomical distribution of the parathyroid glands with the thyroid lobes reflected anteriorly. (Adapted from ref. 13.)

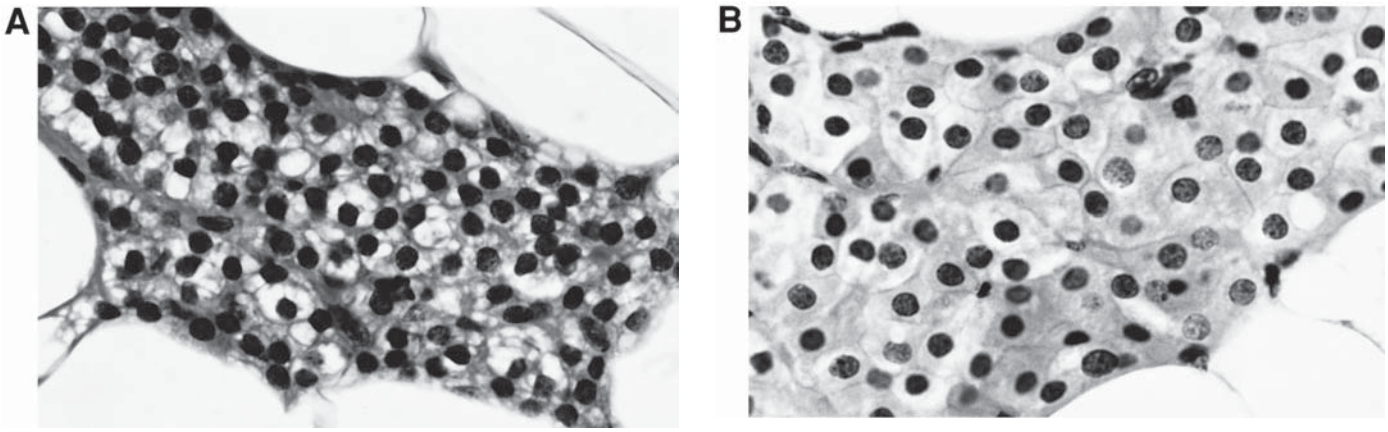

Figure 3 Normal adult parathyroid gland. (**A**) Solid sheet of chief cells with clear vacuolated cytoplasm and regular hyperchromatic nuclei (H&E, original magnification ×720). (**B**) Oxyphil cells with well demarcated granular cytoplasm and regular nuclei (H&E, original magnification ×720).

individuals with chronic diseases [18]. Chief cells are mainly arranged in a solid pattern surrounded by a delicate blood capillary framework. With increasing stromal fat content the chief cells may form anastomosing trabeculae and round to angular nests. Chief cells may form small nodules in older people (Fig. 5), as well as glandular structures [9,17,20]. The lumina from the glandular component may contain eosinophilic PAS-positive colloidlike material that lacks the birefringent oxalate crystals characteristically seen in thyroid colloid under polarization. Amyloid can be found in follicular structures from approx 50% normal parathyroids and its frequency increases with age; parathyroid follicles also increase with age [23]. Ultrastructurally, glandular lumina contain amorphous granular material and/or nonbranching 75–100 Å wide amyloid fibers that may also be found in the cytoplasm of chief cells [20,24,25]. A second epithelial component is the oxyphil cell, which measures up to 50 μm in diameter and shows a polygonal well-demarcated abundant eosinophilic granular cytoplasm with a round less dense and larger nucleus than chief cells (Fig. 3). Less eosinophilic transitional cells may occur [9]. At ultrastructure, oxyphil

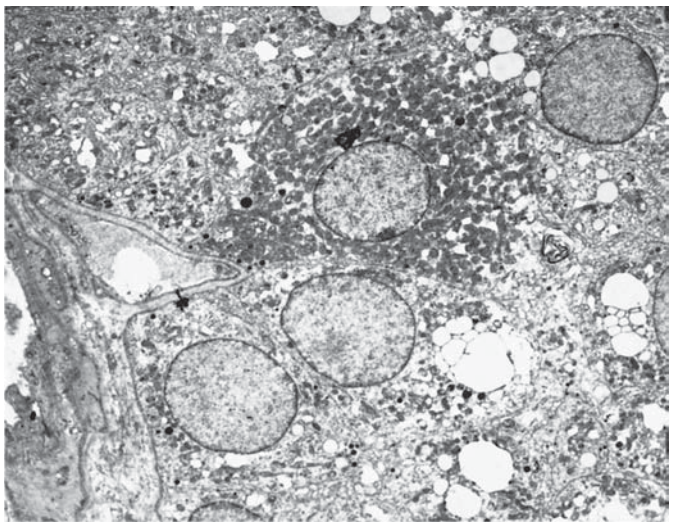

Figure 4 Electron micrograph showing chief cells containing cytoplasmic secretory granules and fat droplets. Note solitary oxyphil cell with abundant intracytoplasmic mitochondria (original magnification ×9500).

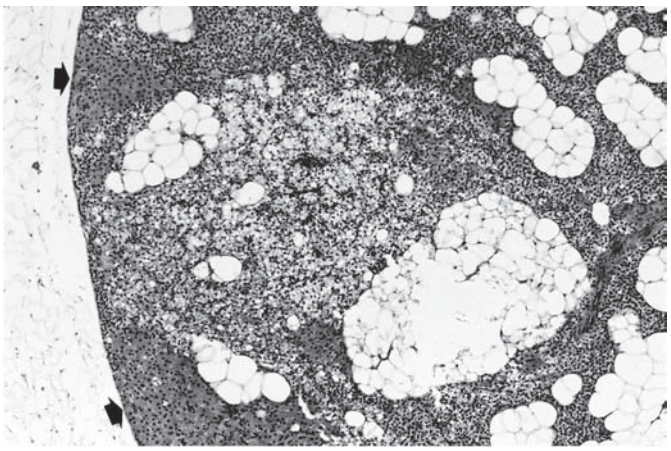

Figure 5 Parathyroid gland from a 66-yr-old female showing an almost equal distribution of parenchymal cells and fatty tissue. Note age-related poorly defined nodular conglomerates of clear vacuolated chief cells and oxyphil cells (*arrowheads*) (H&E, original magnification ×80).

cells show abundant mitochondria and fewer organelles and cytoplasmic membrane-bound argyrophil granules than chief cells [20–22] (Fig. 4). Oxyphil cells increase in number with age (Fig. 5), may be found singly or in small clusters from childhood to adulthood or forming nodular conglomerates difficult to distinguish from oxyphil microadenomas in older persons [9,12], and may acquire a follicular pattern often containing intraluminal amyloid [9,23]. It is estimated that oxyphil cells and transitional cells reach up to 1% of the parenchymal mass in individuals under 40 and approx 5% in older people [12,17].

PTH product and PTH mRNA content show an inverse relationship in normal parathyroid chief cells [26]. Chromogranin A is synthesized by normal parathyroid cells [27] and stored with PTH in the same membrane-bound secretory granules [28]. Normal parathyroid epithelial cells express other molecules, including PTH-related protein, cytokeratins, and calcium-sensing receptor [29–31]. No proliferative cell activity is evident by Ki-67 immunocytochemistry [32–34] and the silver-stained nuclear organizer region (AgNOR) technique [35].

The parathyroid stromal fibrofatty component increases with age, is often irregularly distributed within the parathyroids, and not infrequently occupies the polar regions of the glands, a detail to bear in mind when a biopsy is taken from the poles of the glands. In practice an adult parathyroid gland is considered as normal when it contains up to 50% of stromal/fatty tissue on the sections, but an objective estimate of function can be obtained by planimetric or density gradient studies [12]. The parenchymal weight and total glandular weight in individuals under 18 are almost the same. The mean parenchymal weight in adults remains almost constant throughout life and is approx 75% of the total glandular weight; the amount of stromal/fatty tissue is about 30% depending on age and constitutional body fat. There is a wide variation in the amount and distribution of fatty tissue within a single gland, and also in weight between glands in the same individual and among individuals of the same age. An increase in the parenchymal cell weight was noted in hospitalized as compared with healthy ambulatory subjects, in whites as compared with black people [12,14–17], and with increasing severity of nephrosclerosis in individuals with no clinical evidence of renal disease [36]. Other occasional findings in adult parathyroids include cystic structures, scars or fibrous hyalinizing tissue, and bone marrow tissue, especially in infants [9,12,17].

PHYSIOLOGY OF CALCIUM METABOLISM PTH is synthesized in the chief cell through different processes before being secreted to the bloodstream by exocytosis (Fig. 6). Intact human PTH consists of an 84-amino-acid single-chain polypeptide with a molecular weight of approx 9600; the 1–34 N-terminal fragment exhibits most of the function. Hypocalcemia is the main secretagogue of PTH mediated through the calcium-sensing receptor on the chief cells, which also has *per se* a calcium ion down- or up-regulation effect in the renal tubule. The main effects of PTH are achieved in the kidney and bone through the PTH1 receptor. In the kidney, PTH increases tubular calcium resorption and inhibits phosphate resorption [5]. In bone, PTH activates osteoblasts, which, through release of paracrine factors, activate osteoclasts to bone resorption [37], thus increasing calcium and phosphate in the extracellular fluid and blood. PTH-related peptide is a homolog nonhormone product of PTH. It activates the PTH1 receptor; is ubiquitously synthesized in normal nonendocrine and endocrine tissues, including the parathyroid; and its secretion is not regulated by serum calcium [5]. In physiological conditions it has a predominantly autocrine/paracrine function, including effects on smooth muscle relaxation and milk production during lactation. PTHrP exerts a PTH-like endocrine effect during fetal development and in some pathological conditions when produced in high amounts, such as sarcoidosis [38] and humoral hypercalcemia related to malig-

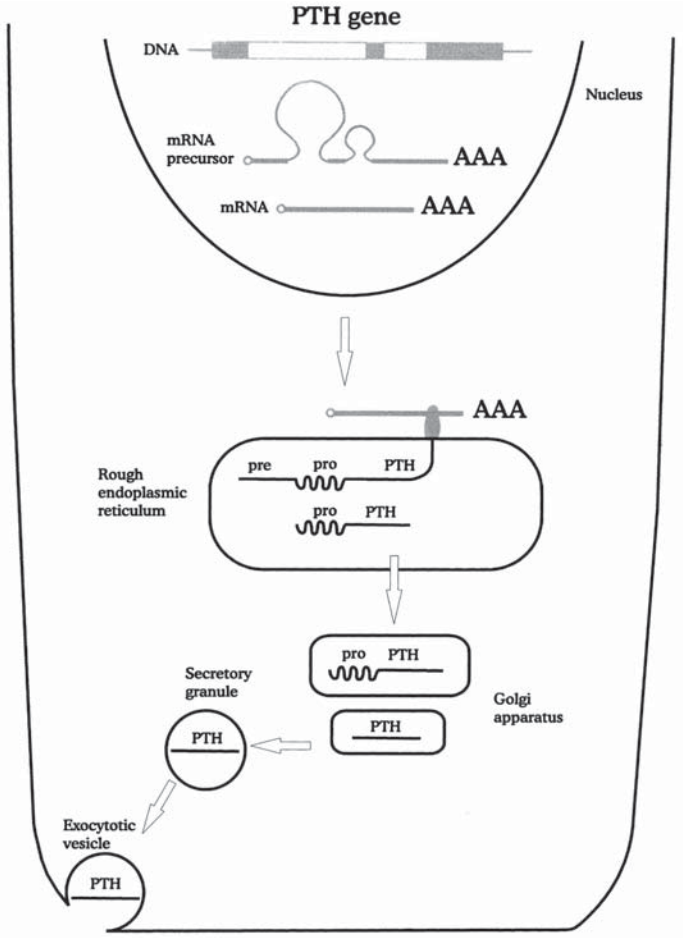

Figure 6 Sequential steps of PTH biosynthesis in parathyroid chief cell.

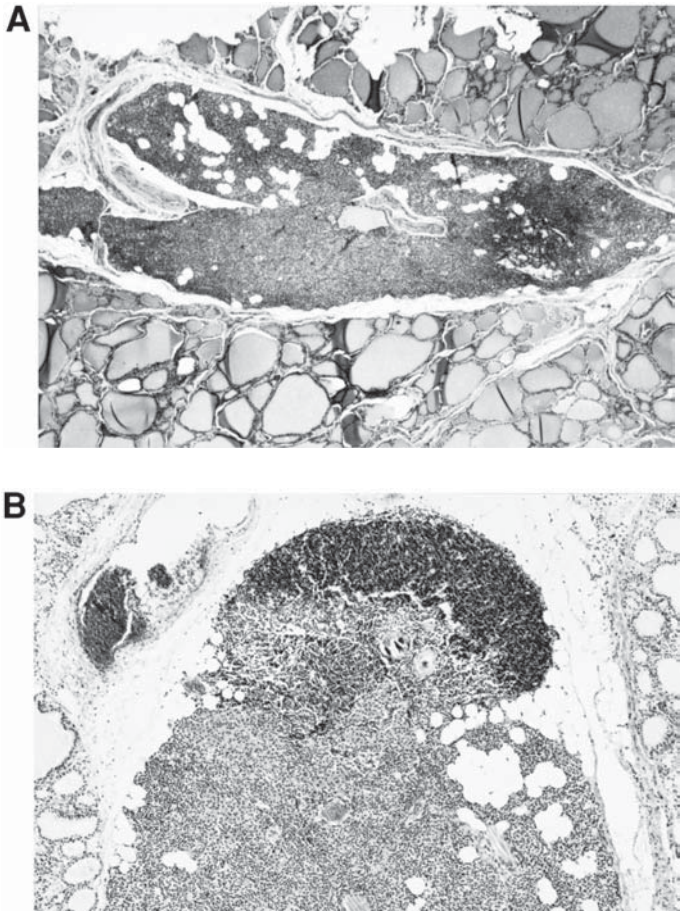

Figure 7 (**A**) Intrathyroid parathyroid gland in a 35-yr-old female aged 35 (H&E, original magnification ×20). (**B**) Intrathyroid parathyroid gland fusioned to thymic tissue from a patient with Graves' disease (H&E, original magnification ×60).

nant and benign tumors [39,40]. Hyperparathyroidism due to ectopic secretion of PTH by a nonparathyroid tumor is rare [41].

Vitamin D and its metabolites 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D, acting through vitamin D receptors, down-regulate the synthesis of PTH in the parathyroid chief cells, increase the absorption of calcium and phosphate in the small intestine, and, simultaneously with PTH, activate bone mineral resorption [42]. Hypercalcemia due to dysregulated high vitamin D production by inflammatory cells and granulomas has been described in sarcoidosis [43], *Cryptococcus neoformans* infection, and by neoplastic cells in various types of lymphoma [44]. No significant morphological abnormalities were found in the parathyroid glands from patients suffering from nonparathyroid hormone related hypercalcemia due to sarcoidosis, vitamin D toxicity, lymphoma, and other malignancies [45].

DEVELOPMENTAL AND ACQUIRED ABNORMALITIES

SUPERNUMERARY PARATHYROID GLANDS Supernumerary (five or more) parathyroid glands were reported to occur in up to 13% individuals [12,13,46]. These should not be confused with small rudimentary glands close to a normal gland or single glands divided by deep lobulations [12]. Proper rudimentary glands located far from the other four glands were reported in up to 5% of individuals at autopsy [12,13]. Most patients with supernumerary glands have five parathyroids, although up to 12 glands have been described. As supernumerary glands are usually associated with the thymic tongue, thymectomy should be advocated in patients with persistent hypercalcemia after surgical resection of four normal or hyperplastic parathyroid glands [11–13].

ECTOPIC PARATHYROID TISSUE Ectopic parathyroid tissue can be situated in areas that are within the normal developmental pathway of the parathyroid glands such as the pharyngeal wall, at or lateral to the carotid sheath, esophageal wall, retroesophageal area, thyroid, thyrothymic ligament, and most frequently the mediastinum, particularly thymus III as a result of failure of separation during development (Fig. 7) [9–13,47,48]. An excessively enlarged hyperplastic or neoplastic parathyroid may lead to further caudal migration of the gland [49]. Parathyroids IV may also be found fusioned to, or embedded in, thymic tissue IV around or within the thyroid [7] (Fig. 7). Occasionally, maldeveloped small nests of parathyroid tissue can be found in such ectopic locations. Heterotopic parathyroid tissue occurring together with thyroid in the vaginal wall has been reported [50]. Any lesion that affects primarily the

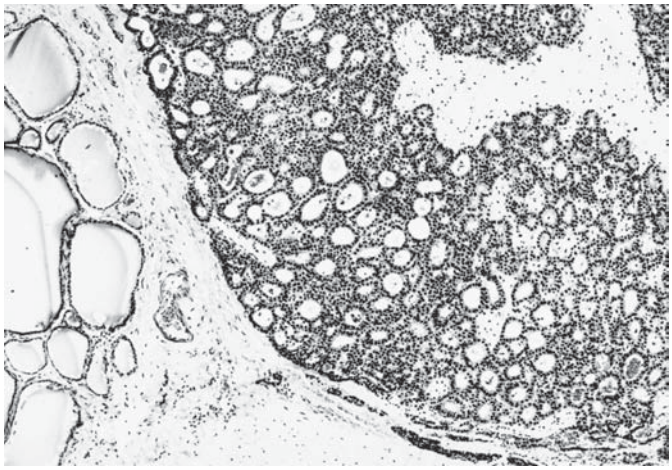

Figure 8 Intrathyroid parathyroid chief cell adenoma entirely composed of follicular structures that immunostained positively for PTH and negatively for thyroglobulin. Note tiny nests of normal looking parathyroid parenchymal tissue at the periphery of the tumor (H&E, original magnification ×30).

parathyroids may occur in ectopic parathyroid tissue and produce hyperparathyroidism [46,49,50–55]. The most common situation during surgery is that in which three normal or hyperplastic parathyroid glands are a found and a detailed search has failed to encounter the "missing" hyperfunctioning gland. Ectopic sites to be explored in these circumstances include the carotid sheath in full extent and the thymus to search for the missing inferior parathyroid gland. An undescended parathymus might leave an inferior parathyroid high in the neck above the superior thyroid poles even at the level of the jaws. If the search failed, the possibility of an intrathyroidal parathyroid lesion should be considered and the thyroid lobe homolateral to the missing parathyroid incised to enucleate the tumor or even proceed to thyroid lobectomy [49,56]. Ectopic parathyroid tissue is easier to find in patients with parathyroid hyperplasia (36% of the cases) [46] than in cadavers (up to 11%) [11–13] as the glands are obviously larger.

Ectopic parathyroid tumors or hyperplasias, as their primary counterparts, may show a wide range of morphological features that can be difficult to differentiate from tumors that occur in the thyroid (Fig. 8). These include follicular adenoma and carcinoma; oxyphil (Hürthle) cell, clear cell, and hyalinizing trabecular tumors; and C-cell neoplasms. Immunocytochemistry for thyroglobulin, calcitonin, and thyroid transcription factor 1 (TTF1) will favor their thyroid origin. PTH immunocytochemistry will help in confirming a diagnosis of an intrathyroid parathyroid tumor [57]. Polarization may prove helpful to identify birefringent oxalate crystals that may be seen in thyroid colloid or amyloid fibers that may occur in parathyroid follicles [58,59]. Intrathyroidal parathyroid adenomas with a dominant papillary architectural component may give rise to a differential diagnosis with thyroid papillary carcinoma [60] which, in contrast, shows classic nuclear features and psammoma bodies.

Another neck tumor that may pose problems in differential diagnosis with parathyroid lesions is the paraganglioma, which shows nests of chromogranin A immunoreactive chief cells surrounded by S100-protein-positive substentacular cells. Metastatic cervical lymph nodes can be replaced by a variety of malignancies that may resemble a parathyroid lesion, including thyroid carcinoma and renal cell carcinoma. Other neck structures that may be interpreted as parathyroid tissue during surgery include lymph node, thymic tissue, paraganglia, salivary gland remnants, accessory thyroid tissue/nodule detached from the main thyroid gland, and lipoma, which should always be carefully analyzed to avoid overlooking a parathyroid lipoadenoma. PTH immunocytochemistry will help in differential diagnoses.

PARATHYROMATOSIS Parathyromatosis refers to hyperfunctioning ectopic or surgically implanted parathyroid tissue nests scattered throughout fat, skeletal muscle, or fibrous tissue of the lower part of the neck and superior mediastinum after parathyroidectomy [61]. Another situation occurs when the capsule from a parathyroid gland is absent and hyperfunctioning peripheral islands of parathyroid epithelial cells lie in fibrofatty tissue. Failure of removal of such tissues in operations for primary or tertiary hyperparathyroidism may lead to persistent hypercalcemia or recurrent hyperparathyroidism. In post-parathyroidectomy implants, seeded parathyroid cell nests can be found lying within fibrofatty tissue that may be adjacent to suture granulomas [61,62] or birefringent talc or starch particles from the initial operation. Other organs that must be considered as a source of ectopic parathyroid rudiments are the vagus nerve [63], vagal ganglion tissue [47], and the thyroid [58]. Differential diagnoses include infiltrating islands of parathyroid carcinoma [61] and paraganglia. The latter shows chromogranin A immunoreactive/PTH negative paraganglionic chief cells surrounded by S100-protein-immunoreactive sustentacular cells [63].

PARATHYROID GRAFTS Parathyroid grafts are made by the surgeon after total thyroidectomy or in patients with tertiary hyperparathyroidism to avoid hypoparathyroidism. The grafts show a histological spectrum that usually correlates with functional phosphocalcic status of the patient, ranging from normal appearing parathyroid tissue to diffuse and nodular hyperplasia due to recurrent tertiary hyperparathyroidism [55]. In this situation, both chief cell and oxyphil cell nodules were found to highly express PTH mRNA [64]. Hyperplastic areas can be seen admixed with striated muscle fibers and fibrofatty tissue resembling invasion (Fig. 9). Development of a parathyroid carcinoma in a parathyroid implant may occur and absolute criteria of such a diagnosis include vessels or nerve fibers invasion and/or metastasis [65]. The presence of nodules, high mitotic count, and high proliferative cell fraction in the orthotopic hyperplastic parathyroids predicts an increased risk of recurrent hyperparathyroidism after autotransplantation [55,66,67].

PARATHYROID CYST True parathyroid cysts are presumed to be the result of excessive and confluent dilatation of parathyroid follicles or canalicular remnants of the embryonic duct (Kursteiner channels) that connects the anterior (or parathyroidal) and posterior (or thymic) ends of the third and fourth pharyngeal pouches [6,68]. Parathyroid cysts predominate in females with a wide age range at presentation, are usually asymptomatic, and often present as palpable solitary cold thyroid nodules. Intracystic hemorrhage may lead to a painful neck swelling [68–71]. The diagnosis of a parathyroid cyst can be achieved by ultrasound-guided fine needle aspiration. The aspirated fluid is usually water

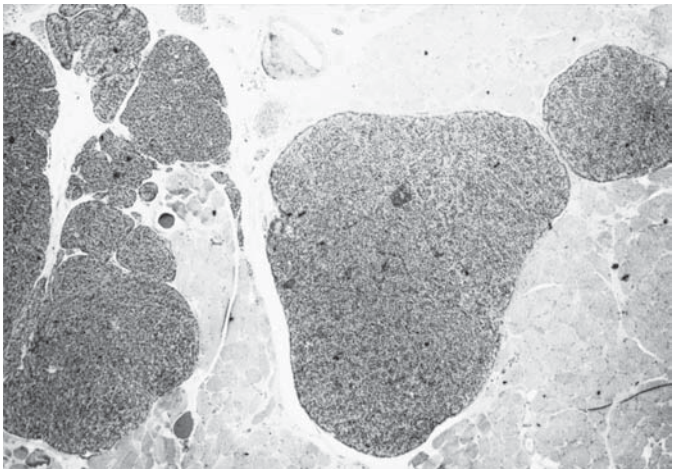

Figure 9 Chief cell nodular hyperplasia admixed with skeletal muscle fibers resembling invasion in parathyroid graft in the arm from a patient with recurrent tertiary hyperparathyroidism (H&E, original magnification ×72).

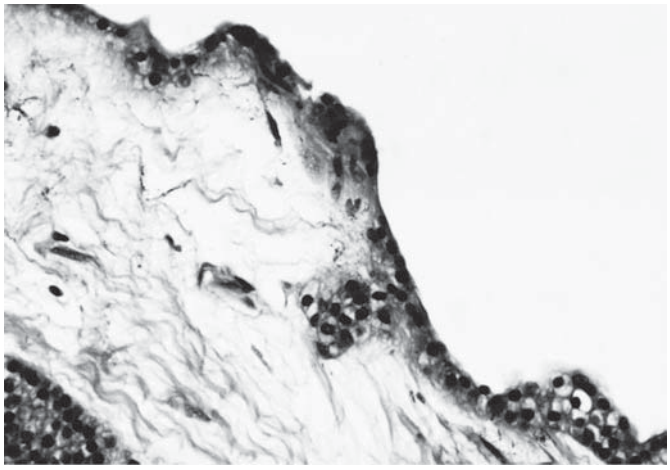

Figure 10 Parathyroid cyst showing a wall lined by single to stratified flat and cuboidal chief cells (H&E, original magnification ×360).

clear or thin straw colored unless hemorrhage occurs, and shows high levels of PTH [72]. Parathyroid cysts may be up to 10 cm in diameter; are usually uniloculated containing transparent, yellowish, or hemorrhagic fluid; and show a semitransparent thin or thick fibrous capsule. Histologically, they may show a rim of normal parathyroid tissue around the capsule or nests of chief cells lying within the cyst wall that is usually lined by single or stratified cuboidal to flattened epithelium [68,69,73] (Fig. 10).

Differential diagnosis between parathyroid cyst and cystic parathyroid adenoma may be difficult on purely morphological grounds. Most parathyroid cysts are thought to develop from preexisting adenomas that underwent infarction or hemorrhage. A cystic parathyroid lesion together with hyperparathyroidism would favor a diagnosis of adenoma. Histological differentiation of cystic parathyroid adenomas from non-neoplastic parathyroid cysts may require several sections to detect or exclude a tumor tissue remnant. The latter may be even absent due to long-standing compression by intracystic fluid or hemorrhage. Degenerate changes sometimes admixed with nests of neoplastic parathyroid cells can be seen in the walls of infarcted cystic adenomas. Branchial cleft cysts occur in the anterolateral aspect of the neck and show a squamous or pseudostratified columnar epithelial cell lining overlying a dense lymphoid infiltrate that may form prominent germinal centers. Intraparathyroidal branchial cleft cysts may occur [74]. Lymphoepithelial cysts show similar features to branchial cleft cysts but are instead surrounded by salivary gland tissue and occur within a lymph node of the neck. Thyroglossal duct cysts occur in the midline of the neck between the foramen cecum and the thyroid and are lined by squamous or respiratory type epithelium; the wall of the cyst may contain thyroid follicles, mucous glands, and inflammatory cells, especially when accompanied by a fistulous tract; and the lumen shows yellow to brownish mucinous material. Thyroid cysts are intrathyroidal and often are the result of infarction or hemorrhage of a thyroid nodule or adenoma. The cyst fluid contains high levels of thyroglobulin and thyroid hormones. Occasional thyroid cysts show features of branchial cleft cysts. Metastatic cystic thyroid papillary carcinoma may replace

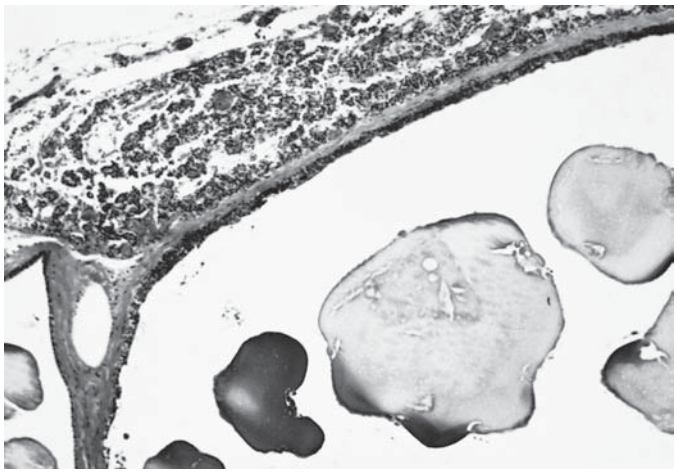

Figure 11 Ultimobranchial cyst from an individual with undescended lingual thyroid showing incomplete fibrous septae and intraluminal mucinous material. Note compressed superior parathyroid gland at the top (H&E, original magnification ×80).

completely a lymph node of the neck and present just as uni- or multiloculated cystic structures lined by a single layer of flattened neoplastic cells showing a minor or no papillary or follicular component. A correct morphological diagnosis can be achieved by careful search for psammoma bodies and the presence of nuclear grooving and cytoplasmic inclusions in the neoplastic cells lining the cyst wall. Unilocular thymic cyst occurs from the angle of the mandible to the manubrium sternum; are lined by flattened, cuboidal, columnar, or squamous epithelium; and the wall usually shows thymic tissue but no inflammatory infiltrates. Ultimobranchial cysts may be found attached to parathyroid IV in individuals with undescended thyroid. These measure up to 1.5 cm in diameter, are often multiloculated, contain intraluminal mucus, show flattened respiratory or squamous epithelial cell lining, and the fibrous septae may contain C-cell conglomerates occasionally admixed with follicular cells (Fig. 11). Cartilage, thymus IV, and salivary gland remnants have been described in association with these cysts [75].

Table 1
Genetics and Pathology of Familial Hyperparathyroidism Syndromes

Syndrome	*Gene*	*Locus*	*Parathyroid histology*	*Other findings*	*Inheritance*
FIHPT-JT	*HRPT2*	1q21–23	Adenoma/carcinoma	Ossifying fibroma of the jaw	AD
FIHPT	*HRPT1*	?	Adenoma/carcinoma	Renal cysts and tumors	AD
MEN 1	MEN 111q13		Hyperplasia	Pancreatic, pituitary, and gut endocrine hyperplasia/tumors Adrenal cortical tumors Lipomas	AD
MEN 2A	*RET*	10q11	Hyperplasia/adenoma	C-cell hyperplasia Thyroid medullary carcinoma Pheochromocytoma	AD
FBHH	*CSR*[a]	3q,19p	Normal Mild hyperplasia Lipohyperplasia		AD
NSHPT	*CSR*[b]	3q	Mild hyperplasia		AD

MEN, Multiple endocrine neoplasia; FHPT-JT, familial hyperparathyroidism-jaw tumor syndrome; FHPT, familial isolated hyperparathyroidism; FBHH, familial hypocalciuric hypercalcemia; NSHPT, neonatal severe hyperparathyroidism; CSR, calcium sensing receptor gene ([a]heterozygous and [b]homozygous inactivating mutations); AD, autosomal dominant.

PARATHYROID APLASIA OR HYPOPLASIA (DIGEORGE'S SYNDROME) DiGeorge's syndrome is an autosomal dominant congenital developmental anomaly involving the third and fourth pharyngeal pouches and is characterized by total or partial absence of the thymus and parathyroid glands. It occurs concomitantly with cardiovascular and craniofacial malformations [5]. Patients suffer from neonatal hypocalcemic seizures due to PTH deficiency and from severe infections due to thymic aplasia.

PRIMARY HYPERPARATHYROIDISM

Primary hyperparathyroidism refers to an increase of serum calcium and PTH concentrations. The classical presentation such as hypercalcemia accompanied by bone pain due to osteitis fibrosa cystica (von Recklinghausen's disease) and symptoms of renal lithiasis showed a remarkable decrease after the development of routine serum calcium screening. This changing laboratory methodology in the late 1960s was responsible for about a fivefold increase of primary hyperparathyroidism presenting with mild hypercalcemia, either asymptomatic (approx 50% of the cases) or with additional vague symptoms such as muscular weakness, malaise, arterial hypertension, mental depression, and mild osteoporosis. Sporadic primary hyperparathyroidism predominates in women in a proportion of 3:1; the average age at diagnosis is 55 yr. The incidence of primary hyperparathyroidism after the introduction of calcium screening in a population-based study of postmenopausal women was 112 per 100,000 in 1974, and then fell to 8 per 100,000 in 1992, a decline attributed to prior removal of tumors in patients whose hypercalcemia was discovered at a younger age [5,76, 77]. Diffuse or nodular parathyroid gland enlargement is found in about 1% of thyroidectomized female patients, probably representing early normocalcemic subclinical forms of hyperparathyroidism. The parathyroid glands are usually smaller than those found in patients with preoperatively proved primary hyperparathyroidism [78,79]. Approximately 10% of patients on lithium therapy for manic–depressive disease become hypercalcemic due to parathyroid adenoma or hyperplasia [80,81]. Primary hyperparathyroidism occurring at a young age should raise the suspicion of antecedents of neck irradiation in childhood with consequent parathyroid tumor generation [82] or a familial syndrome (Table 1). Familial hyperparathyroidism–jaw tumor syndrome is characterized by the association of parathyroid adenoma(s) (often cystic) with ossifying fibroma(s) of the jaw and renal cysts/tumors that may occur individually or in com-bination in one and the same patient usually in childhood or adolescence. Familial isolated hyperparathyroidism shows parathyroid adenomas without associated tumors from other organs.

Molecular studies support the view that a subset of cases represents a variant of familial hyperparathyroidism–jaw tumor syndrome. An increased incidence of parathyroid carcinoma has been described in both syndromes [5,83,84]. MEN 1 is associated with proliferative lesions of the parathyroid glands, pituitary, and endocrine pancreas. Other associations include carcinoid tumors from the foregut and thymus, adrenal cortical tumors, and lipomas. MEN 1 is found in up to 17% patients with primary hyperparathyroidism and in up to 43% with primary parathyroid hyperplasia. Hyperparathyroidism is the most common manifestation and by age 40 its prevalence approaches 100%. The age at diagnosis in screenings is about 20 yr. Hyperparathyroidism is the most common manifestation of MEN 1, which is found in up to 17% patients with primary hyperparathyroidism and in up to 43% with primary parathyroid hyperplasia [84–87]. MEN 2A is associated with thyroid C-cell hyperplasia/medullary carcinoma, pheochromocytoma, and parathyroid hyperplasia/adenoma. The prevalence of parathyroid disease in this setting is approx 20–30%, 0.3% in patients with primary hyperparathyroidism and 1% in cases with primary parathyroid hyperplasia. The mean age at diagnosis of 38 yr is found concomitantly with thyroid medullary carcinoma or pheochromocytoma in 77% of the cases, and may present with single or multiple parathyroid gland enlargement at initial operation [86,88–90]. Familial benign hypocalciuric hypercalcemia is characterized by mild hypercalcemia, high PTH levels, and low urinary calcium excretion, whereas neonatal severe hyperparathyroidism is the

clinically more aggressive phenotype of the disease due to homozygous activating mutation of the calcium sensing receptor gene [5,84,91–93] (Table 1).

PARATHYROID ADENOMA The most common cause of primary hyperparathyroidism (80% or over) is the parathyroid adenoma. It occurs two to three times more frequently in females and shows a wide age range with a higher frequency in the fifth and sixth decades [51,61,73,85,86,94]. The larger tumors are more frequent in patients with bone disease than those without, and in patients with higher serum calcium and PTH levels [34,36,73,94]. Adenomas have a tendency to be smaller than in the past since the introduction of calcium screening [51,73,76,95]. Unless cystic, adenomas are rarely large enough to be detected as a palpable mass or produce local compressive symptoms [51]. Infarction of an adenoma, either spontaneous or after ultrasound-guided fine needle aspiration, may lead to local painful neck swelling due to acute hemorrhage and spontaneous remission of the hyperparathyroidism [96]. In a large unselected autopsy series, the prevalence of parathyroid chief cell adenoma was 2% [36]. Parathyroid adenomas show an increased incidence after low-dose external neck irradiation and have been reported after radioiodine therapy for Graves' disease [74,75]. A relation between dose–response and generation of parathyroid adenomas has been noted [82] with a latency period for their clinical manifestation being about 30–40 yr [97,98]. Radiation-associated parathyroid adenomas show similar clinicopathological features to the sporadic ones, except that the patients manifest a higher frequency of thyroid neoplasms [53] and are usually younger at diagnosis [82], generally due to radiation exposure in childhood. Parathyroid adenomas occurring in young people should also raise the suspicion of a genetically determined defect [83]. The majority of sporadic parathyroid adenomas are monoclonal or oligoclonal, while a minority show mutations of the *cyclin D1*, *MEN1*, *p53*, and *GNAS1* genes, respectively, and frequent (25% or more) loss of heterozygosity on chromosomes 1p, 6q, 11q, and 15q [84,99].

Parathyroid adenomas are more common in the lower parathyroid glands and vary considerably in size (from a few millimeters to 10 cm) and weight (0.025–120 g) [51,73,95,100]. They are ovoid, elongated, or lobulated; usually have a soft consistency; vary from tan to orange-brown in color, or bluish if toluidine blue is used to aid surgical localization; and most cases show a homogeneous cut surface with or without evident nodularity. Cystic changes may occur. A rim of normal parathyroid tissue is sometimes macroscopically evident around the tumor [51,73] (Fig. 12). Microscopically, parathyroid chief cell adenomas may show a wide cytoarchitectural spectrum [101] (Figs. 13 and 14). Large pleomorphic nuclei occur in approx 10% of the cases [102] (Fig. 13) and, more rarely, multinucleate chief cells. Oxyphil cells may occur within a chief cell adenoma as forming clonal nodules or admixed with chief cells, which may also grow in a clonal pattern (Fig. 12). The neoplastic cells can be arranged in solid, trabecular, follicular, microcystic, papillary, and peripheral palisading structures, or an admixture [12,51,73,103] (Figs. 13 and 14). Lumina from glandular structures may be empty, show colloidlike material, or contain amyloid [23,25] (Fig. 14). Cystic changes in an adenoma may be genuine (Fig. 13) or occur after tumor infarction

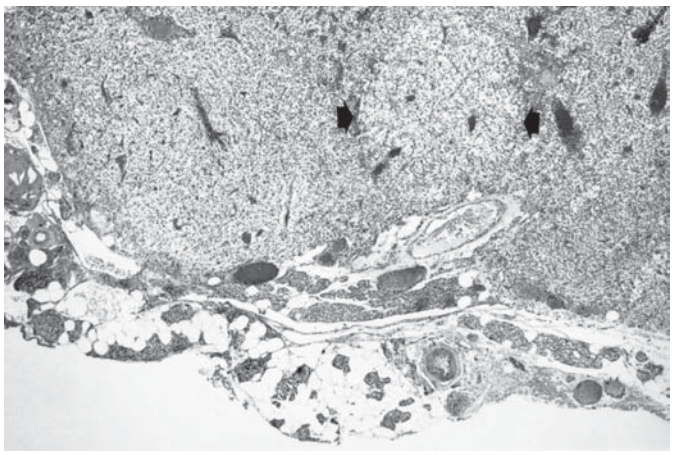

Figure 12 Unencapsulated parathyroid adenoma with a rim of normal parathyroid tissue. Note the central small clonal nodule of chief cells with clear vacuolated cytoplasm (*arrows*) (H&E, original magnification ×20).

(Fig. 15), fine needle biopsy, or parathyroidectomy by percutaneous ethanol injection [104,105]. Adenomas may show a high mitotic rate [51,106], particularly noted in cases with hyperparathyroid crisis [107]. The stroma of parathyroid adenomas is often delicate fibrovascular, although edematous, myxomatous, fibrohyalinizing, and fatty tissue may occur. Degenerate changes that could be present in parathyroid adenomas include necrosis, calcification, ossification, cholesterol granulomas, and inflammatory cells [51,73]. A rim of "normal" parathyroid tissue can be found around the tumor capsule or merging with neoplastic cells (Fig. 12), or may not be unidentifiable when the gland is totally replaced by tumor [73]. Often chief cells from the "normal" supressed parathyroid tissue are slightly smaller with crowded nuclei, contain abundant PAS-positive glycogen granules that are also identifiable at ultrastructure [20,108], and show prominent PTH storage with low PTH mRNA expression [26]. Sudanophilic or oil red O positive intracytoplasmic fat globules are readily visible on frozen sections and at ultrastructural levels, and are usually scarce or absent in adenomas due to increased cell activity [19,20,108]. Cells from parathyroid adenomas show a varying extent of argyrophilia, chromogranin A and PTH-immunoreactivity, and PTH mRNA expression [21,26,27]. Ultrastructurally, the adenomatous cells show features of chief cell differentiation, including the presence of membrane-bound secretory granules [43,86].

The main differential diagnoses of parathyroid adenoma are parathyroid carcinoma—which shows evidence of invasion or metastasis, and parathyroid nodular hyperplasia—which shows multiple gland involvement. The occurrence of adenomas involving two or more glands is rare. In this case the possibility of antecedents of neck irradiation or a genetically determined defect [82,83,88] must be considered.

VARIANTS OF PARATHYROID ADENOMA

Cystic Adenoma This variant may be difficult to differentiate from a parathyroid cyst and should not be confused with ordinary parathyroid adenomas that may contain a minor cystic

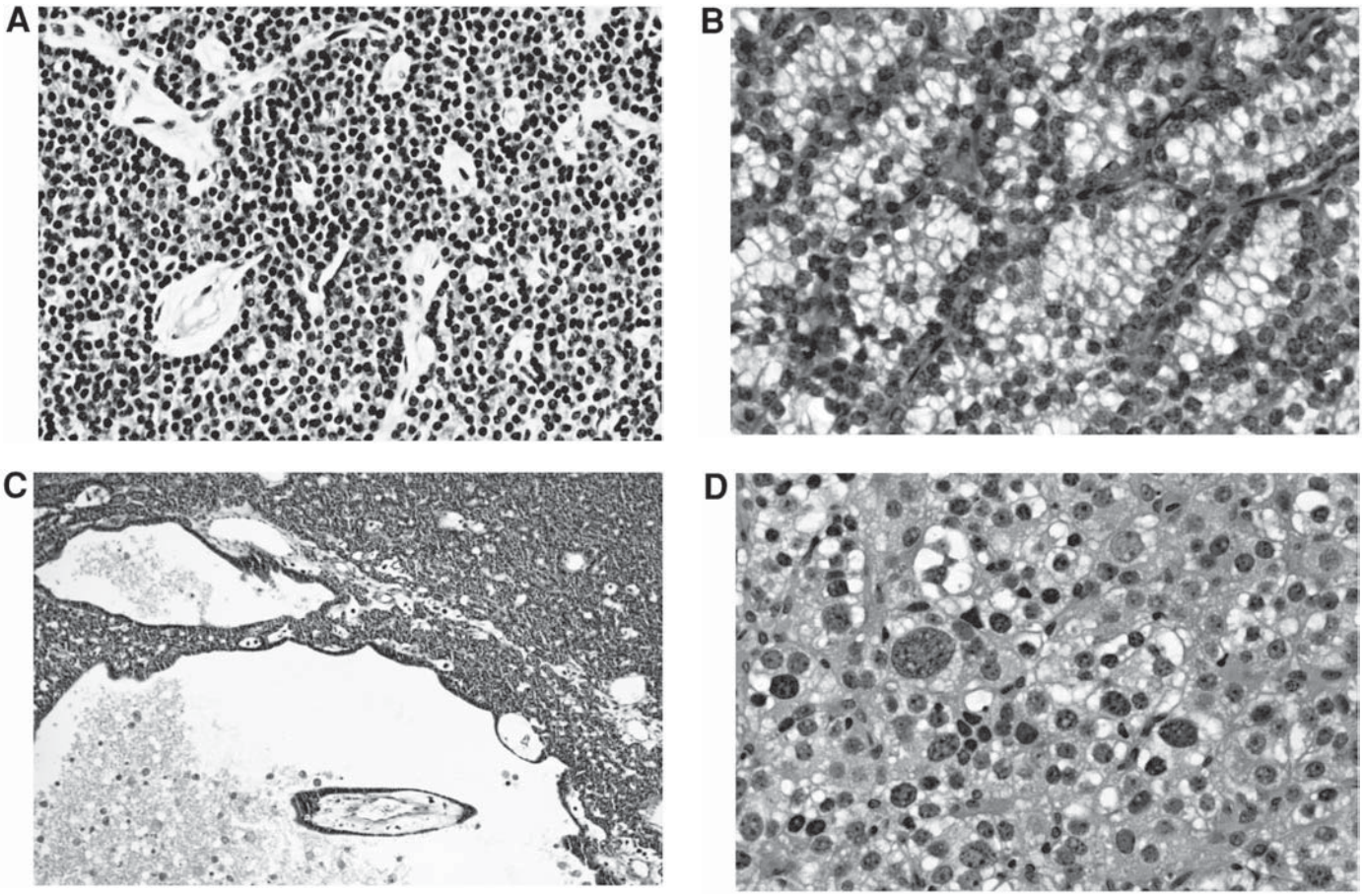

Figure 13 Parathyroid chief cell adenomas showing (**A**) small dark cells with round regular nuclei arranged in a solid pattern and admixed with delicate fibrovascular stromal tissue; (**B**) trabeculae of clear vacuolated cells with round regular nuclei; (**C**) small cells forming nests, follicles, and microcysts (H&E, original magnification ×180); and (**D**) cells with nuclear pleomorphism (H&E, original magnification ×360).

component. Adenomas can be originally cystic as illustrated by their occurrence in familial hyperparathyroidism–jaw tumor syndrome, for example [83], although most cystic adenomas probably originate as the result of infarction and consequent hemorrhage in a preexisting adenoma [68] (Fig. 15). The adenomatous origin of the lesion should be suspected when symptoms of hyperparathyroidism exist, although parathyroid cystic adenomas may become asymptomatic when tumor tissue has undergone infarction or been compressed by expanding intracystic fluid or hemorrhage. Cystic adenomas predominate in females, show a wide age range at presentation, and often present as a palpable solitary cold thyroid nodule. The patient may present with a painful neck swelling due to intracystic hemorrhage [68–70,72]. Hyperparathyroid crisis may occur after massive release of PTH due to necrosis of an adenoma. Cystic adenomas can measure several centimeters, are usually uniloculated, and show a thick capsule that may contain a grossly identifiable focal brownish area of neoplastic parathyroid tissue. Histological identification of adenomatous cells may require several sections from the cyst wall to detect tumor tissue remnant [68,69,73]. Diagnosis, management, and differential diagnoses of parathyroid cystic adenomas are basically the same as for parathyroid cysts (*see* earlier) [68–70].

Oxyphil Adenoma Fewer than 8% of hyperfunctioning parathyroid adenomas are oxyphil in type. The tumors are tan in color and, histologically, most or all neoplastic cells show abundant eosinophilic granular cytoplasm with regular hyperchromatic central nuclei sometimes showing a prominent nucleolus (Fig. 16). Nuclear pleomorphism and multinucleated cells may occur. At ultrastructure, cytoplasmic mitochondria are numerous admixed with few scattered secretory granules. The tumor cells show PTH immunoreactivity and stain for phosphotungstic acid-hematoxylin [51,73,109–111].

Lipoadenoma Parathyroid lipoadenoma may occur with hyperparathyroidism and present as a neck mass. Lipoadenomas vary in weight and measure up to several centimeters. The first reported tumor weighed 420 g and was called a "hamartoma" [112]. Grossly, the tumors are roundish, ovoid, or lobular in shape with a smooth external surface, and show a yellow-tan appearance depending on the amount of fatty tissue component which varies from 20% to even >90%. Lipoadenomas are composed of chief cells, with or without oxyphil cells; and arranged in solid, trabecular, and glandular patterns, or an admixture, surrounded by prominent fatty tissue component resembling the normal adult parathyroid gland (Fig. 17). The stroma may show myxomatous changes, inflammatory cells, and calcium deposits

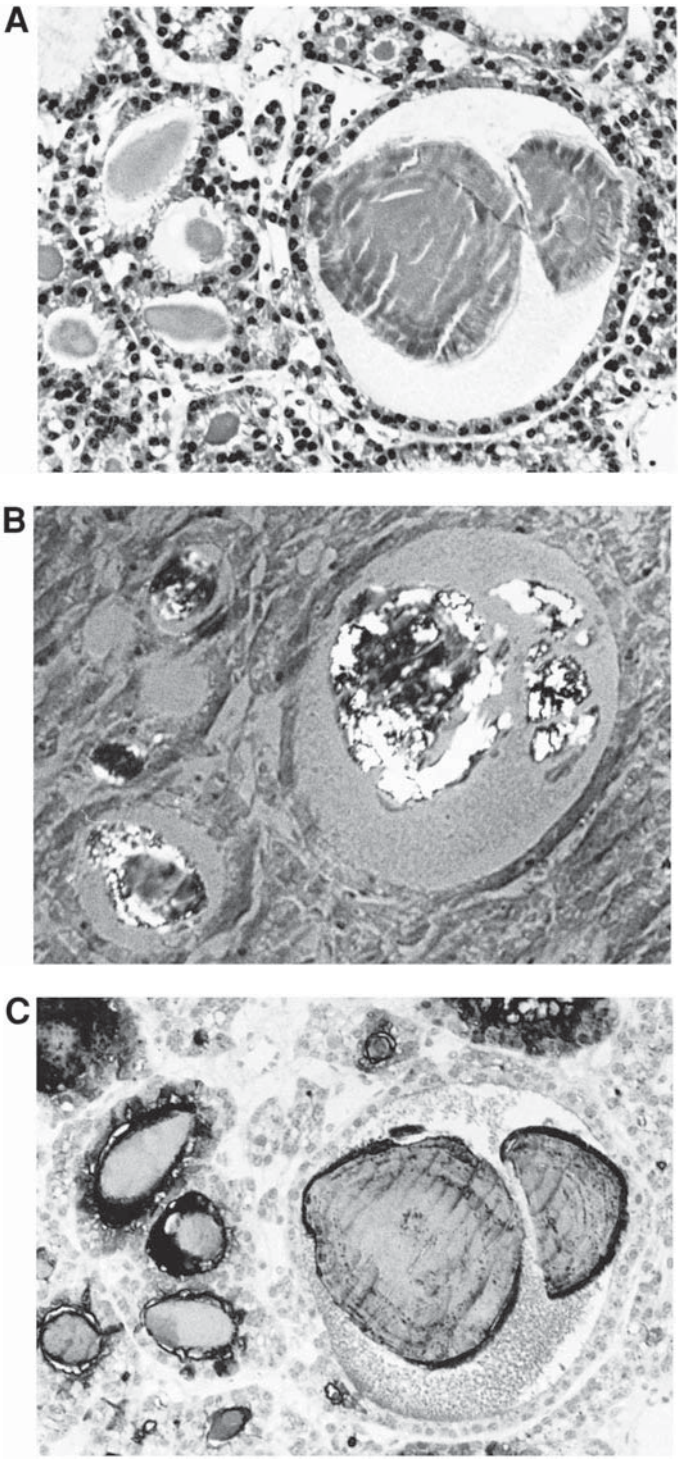

Figure 14 Parathyroid chief cell adenoma showing (**A**) follicular structures with intraluminal colloidlike material and dense eosinophilic amyloid that displays (**B**) apple green birefringence after polarization, and (**C**) PTH-immunoreactivity which is also present in some neoplastic cells (H&E, Congo red, and immunoperoxidase, respectively, original magnification ×450).

[112–115]. Tumors composed entirely or almost entirely of oxyphil cells (oncocytic lipoadenoma) are rare (Fig. 17). One of the differential diagnoses is the lipoma [116] and distinction from lipohyperplasia will depend on the number of glands affected.

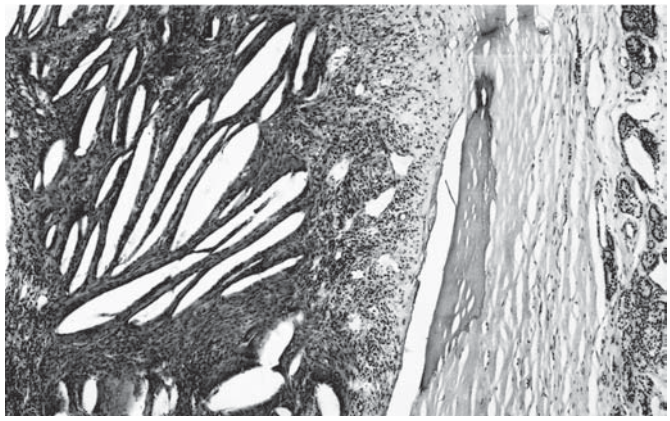

Figure 15 Cystic adenoma showing a thick fibrous calcified capsule around infarcted adenomatous tissue replaced by fibrosis and cholesterol clefts. Note rim of normal parathyroid tissue around the cyst wall (H&E, original magnification ×180).

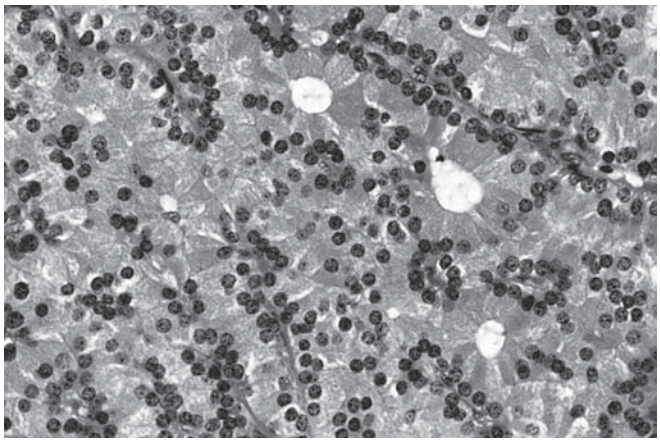

Figure 16 Oxyphil adenoma showing trabecular/solid and glandular structures of well demarcated cells with abundant granular eosinophilic cytoplasm and regular nuclei (H&E, original magnification ×450).

Water-Clear Cell Adenoma This rare tumor shows nests and glandular structures of PTH-immunoreactive microvacuolated cells with abundant well-demarcated cytoplasm (Fig. 18). Ultrastructurally, the cells contain characteristic cytoplasmic empty membrane-bound vesicles [117] as seen in primary water-clear cell hyperplasia [108]. Double water-clear cell adenomas have been reported [118]. A rim of normal parathyroid tissue has been described in primary water-clear cell hyperplasia, making the histological differentiation with adenoma difficult if two or more glands are not examined [119]. Differential diagnoses include parathyroid adenomas composed of optically clear vacuolated chief cells that do not show the classic ultrastructural features of water-clear cells. Other tumors composed of clear cells may occur in the parathyroid region, including metastatic renal cell carcinoma, metastatic thyroid clear cell carcinoma of follicular or C-cell origin, and paraganglioma. PTH, thyroglobulin, and calcitonin immunocytochemistry would play an important role in their differential diagnosis, and between

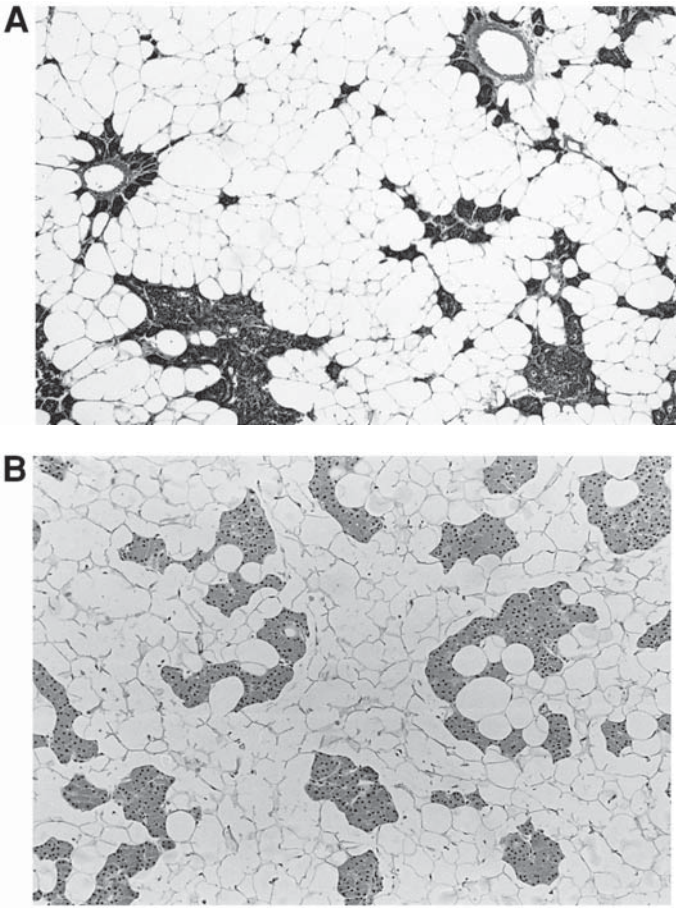

Figure 17 (**A**) Lipoadenoma showing chief cells arranged in nests and glandular structures admixed with prominent adipose tissue component (H&E, original magnification ×80). (**B**) Oncocytic (oxyphil cell) lipoadenoma (H&E, original magnification ×80).

primary thyroid clear cell neoplasms and intrathyroid water-clear cell parathyroid adenomas [120].

Microadenoma Parathyroid microadenomas show an incidence of 1% of all operations for primary hyperparathyroidism and are defined as single lesions smaller than 6 mm that occur in externally undeformed parathyroid glands [95]. This high incidence is probably due to early detection of hyperparathyroidism by calcium screening. Parathyroid microadenomas may be missed on surgical exploration and their search during frozen section may be difficult and laborious [29]. Multiple sections may be required to identify a parathyroid microadenoma in an otherwise normal parathyroid gland. The oil red O method during frozen section, PTH mRNA expression on paraffin-embedded tissue, and correlating the findings with the effect of surgery on serum calcium levels might help to interpret tissue functionality. Microadenomas are composed of nests of chief cells or oxyphil cells that may present as a small micronodule completely or partially surrounded by normal parathyroid tissue, as a diffuse hypercellular lesion involving all the parathyroid gland, or as a poorly circumscribed diffuse hypercellular area showing no clear-cut distinction from adjacent normal parathyroid tissue [95,100].

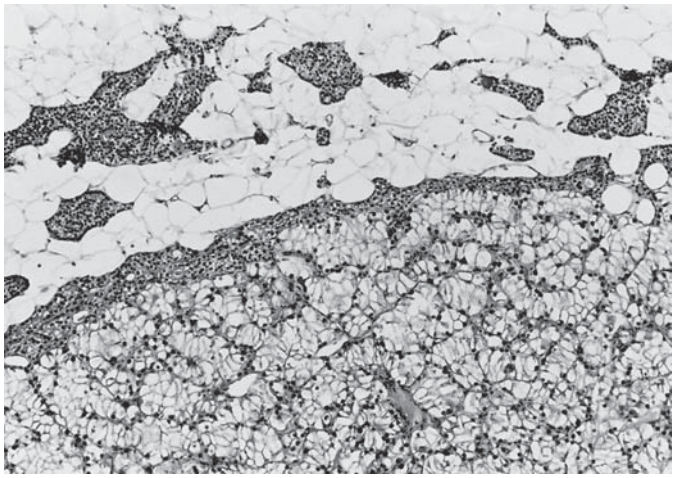

Figure 18 Water-clear cell adenoma showing nests of cells with abundant clear vacuolated cytoplasm containing regular nuclei surrounded by a rim of normal parathyroid tissue (H&E, original magnification ×80).

PARATHYROID CARCINOMA Parathyroid carcinoma accounts for fewer than 5% of cases of primary hyperparathyroidism. Most parathyroid carcinomas are sporadic, but they have also been reported to occur in association with parathyroid hyperplasia in primary and tertiary hyperparathyroidism, with high frequency in familial isolated hyperparathyroidism and familial hyperparathyroidism–jaw tumor syndrome (Table 1), and after neck irradiation. The majority of sporadic parathyroid carcinomas are associated with somatic diallelic mutation of the retinoblastoma (*Rb*) gene and *cyclin D1* overexpression, whereas a few of them may show *p53* mutation. As yet unidentified oncogenes and tumor suppressor genes from several chromosomes seem to contribute to the development of parathyroid carcinoma [121].

Patients usually present with symptoms of severe hyperparathyroidism and show a wide age range with a peak in the fifth and sixth decades with an almost equal sex distribution. A high proportion of cases have a palpable mass in the neck with or without laryngeal nerve palsy and suffer from bone and/or renal disease at diagnosis. Fewer than 5% of the tumors are asymptomatic with hypercalcemia discovered on routine serum calcium screening [121]. Parathyroid cancer may present as painful goiter [122]. At operation the tumors may be found invading the recurrent laryngeal nerve, thyroid strap muscles, thyroid gland, trachea, carotid sheath, or esophagus. Metastases have been reported to occur in up to 32% of the cases—regional lymph node and distant metastases in up to 32% and 24%, respectively. The most frequent distant metastatic sites include lungs and liver. Death is usually related to uncontrollable hypercalcemia. Five-year survival rates vary from 40% to 86%, with a recently reported 10-yr survival rate of 49% in a large series [121,123].

The majority of parathyroid carcinomas weigh between 2 and 10 g, and measure from 1.3 to several centimeters in greatest diameter with a median size of 3.3 cm [54,102,123]. The tumors are ovoid or lobulated in shape, sometimes showing a ragged external surface with adhered fat, striated muscle, fibrous,

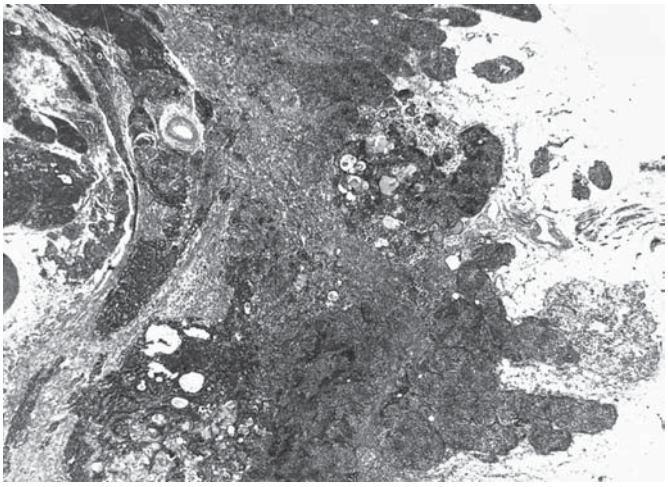

Figure 19 Parathyroid carcinoma showing fibrous septae, irradiating growth pattern, and ragged edges of invasive islands of neoplastic chief cells deeply infiltrating surrounding fibrofatty tissue (H&E, original magnification ×20).

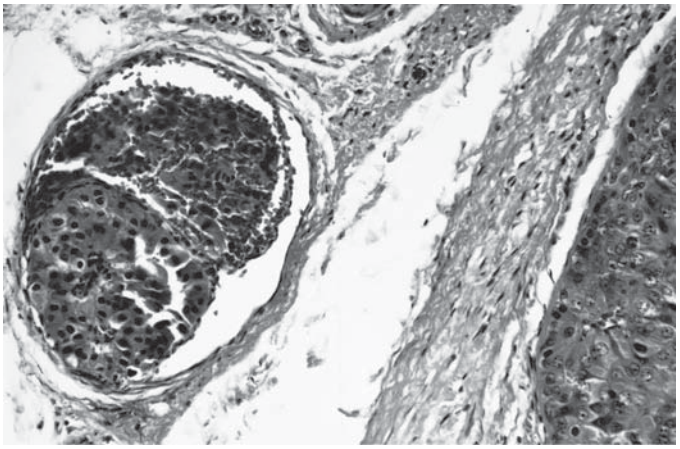

Figure 21 Oxyphil parathyroid carcinoma showing solid sheets of cells with abundant eosinophilic granular cytoplasm, vesicular nuclei, and prominent nucleoli. Blood vessel invasion is present (H&E, original magnification ×600).

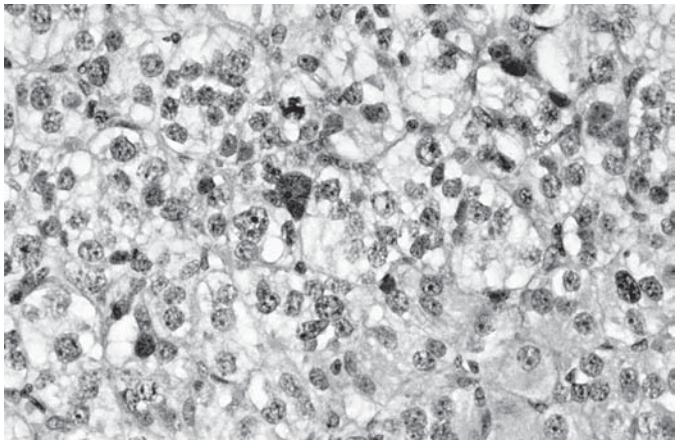

Figure 20 Parathyroid carcinoma showing a solid growth pattern of chief cells with large slightly irregular overlapping nuclei with prominent nucleoli. Note tripolar mitosis (H&E, original magnification ×720).

nerve, or thyroid tissue (Fig. 19). On section, parathyroid carcinoma shows a white to tan soft or hard cut surface with or without calcification or necrosis [54,102]. Histologically, the tumors are composed of classic or optically clear chief cells usually arranged in a solid pattern sometimes forming peripheral palisading. A varying amount of trabecular, follicular, and spindle cell components may occur. Chief cells often show slightly irregular hyperchromatic or vesicular nuclei which are usually larger than those seen in adenomas and contain a prominent nucleolus (Fig. 20). Nuclei may contain cytoplasmic inclusions and show marked pleomorphism. An admixture of chief cells and oxyphil cells may occur [102,124,125]. The mitotic rate is usually high but may be absent. Necrosis may be present. At ultrastructure, neoplastic cells show prominent nucleoli and cytoplasmic features of parathyroid chief cell differentiation often with a small number of secretory granules [20]. Oxyphil parathyroid carcinomas (80% or more oxyphil cell component) do not differ clinically and morphologically from chief cell parathyroid carcinoma, except that the cells show abundant granular eosinophilic cytoplasm (Fig. 21) due to abundant mitochondria content [126,127]. Parathyroid carcinomas usually show dense fibrous septae involved by tumor cell islands, which can also be found in the tumor capsule and invading blood vessels, nerve fibers, and adjacent neck structures [54,102,124] (Figs. 19 and 21). Orcein stain for elastic fibers or endothelial cell markers may prove useful for demonstrating vascular invasion. PTH and/or PTHmRNA expression could be essential for identifying classic, nonfunctioning, metastatic, and unusual types of parathyroid carcinomas [26,128,129] (Fig. 22). Parathyroid carcinoma usually shows negative or predominantly negative Rb protein immunoreactivity, which stains positively parathyroid adenomas [84,121]. p53 expression was found in a few parathyroid carcinomas that showed no Rb mutation, but also in occasional adenomas and in hyperplastic tissue [84,121,130].

Differential diagnosis between parathyroid carcinoma and adenoma may be difficult. Cyclin D1 overexpression, DNA ploidy, AgNOR enumeration, and proliferative rate as shown by Ki-67 may show overlapping results making these markers and methods of little value in their differential diagnosis [35, 121,124]. Absolute criteria of malignancy include lymphatic or vascular invasion, invasion to adjacent neck structures, and regional or distant metastasis. In practice, suspicious features of malignancy would include high mitotic and proliferative rates, broad intratumoral fibrous septae, necrosis, atypical cells with large regular nuclei containing prominent nucleoli, DNA aneuploidy, and no Rb protein immunoreactivity. In the absence of invasion or metastasis, a diagnosis of "parathyroid adenoma with suspicious features" or "borderline parathyroid tumor" should be made and close follow-up advocated. In borderline cases the surgical report of fixation of the tumor to surrounding structures of the neck would support a diagnosis of malignancy. Pathologists must be aware that necrosis, bands of fibrosis, adherence to surrounding neck structures, and even laryngeal nerve

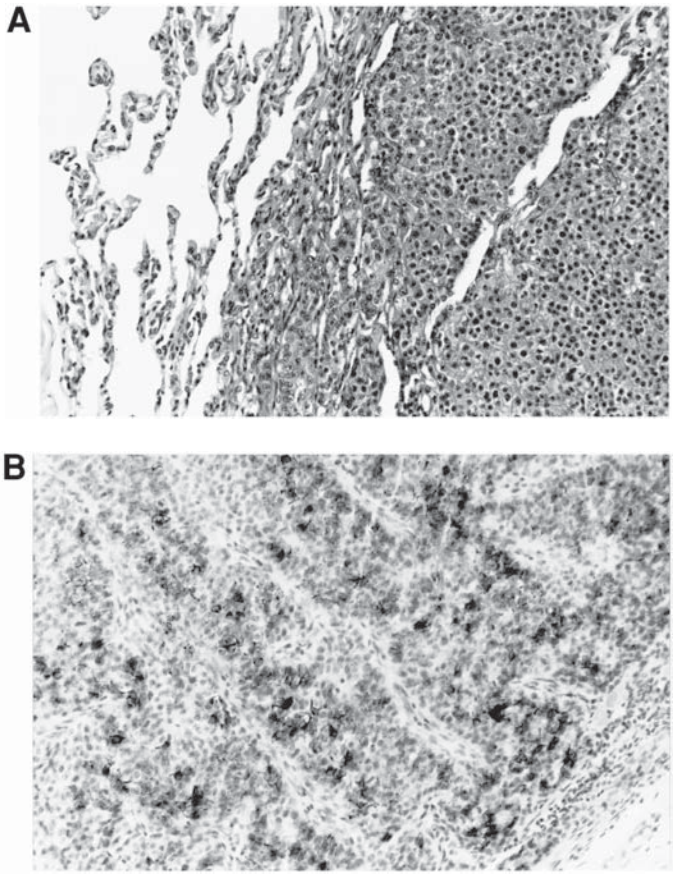

Figure 22 (**A**) Parathyroid carcinoma metastatic to the lung (H&E, original magnification ×450) showing (**B**) patchy PTH immunoreactivity (Immunoperoxidase, original magnification ×450).

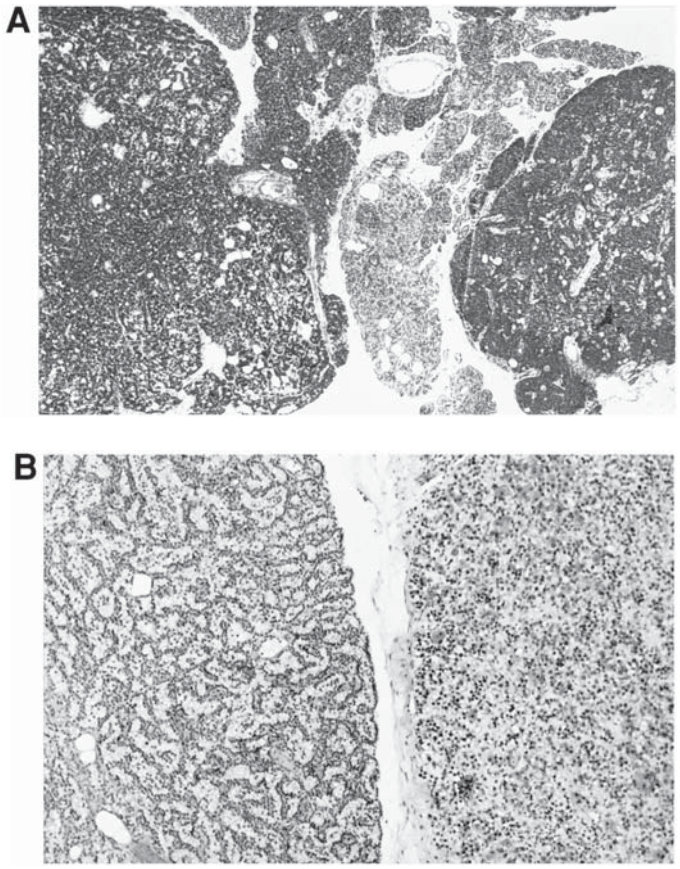

Figure 23 (**A**) Parathyroid gland from a patient with MEN 1 showing cytoarchitectural pleomorphism with multiple nodular component admixed with diffuse hyperplastic areas. To the middle low a few adipose cells are seen admixed with hyperplastic tissue resembling normal parathyroid (H&E, original magnification ×20). (**B**) Well demarcated chief cell nodules with varying cytoarchitectural features in sporadic primary nodular hyperplasia (H&E, original magnification ×72).

palsy may occur in parathyroid adenomas following therapeutic percutaneous ethanol injection [104,105]. Other differential diagnoses where immunocytochemistry would stand include paraganglioma, metastatic renal cell carcinoma, and some thyroid C-cell, follicular cell and oxyphil (Hürthle) cell carcinomas metastatic to neck lymph nodes.

PARATHYROID HYPERPLASIA

PRIMARY NODULAR HYPERPLASIA Parathyroid hyperplasia is the cause of primary hyperparathyroidism in approx 15% of the cases and shows a female:male ratio of 2–3:1 with a wide age range at presentation and the highest frequency after the fifth decade [85,86]. Cystic parathyroid hyperplasia may present clinically as a neck mass [70]. Primary parathyroid nodular hyperplasia may occur as a sporadic disease, after neck irradiation, or as a component of multiple endocrine neoplasia syndromes [85,87,89,98,131]. Parathyroid hyperplasia is polyclonal with monoclonality usually restricted to nodular areas [84]. All four parathyroids are usually enlarged; however, owing to early diagnosis by serum calcium and family screening (as in MEN 1 and MEN 2A patients), the extirpated glands may show normal histology in some cases [85,86,131–133].

The combined weight of four primary hyperplastic parathyroid glands ranges between 0.1 and 25 g with a gland size of up to several centimeters. The parathyroids are usually ovoid with lobulations and occasional pseudopodal projections. The cut surface is homogeneous, often nodular, and tan to dark red in color. Cysts with clear yellow or transparent fluid may occur [4,85,131,133]. In general, the upper glands are slightly larger than the lower [4], except for MEN 1 parathyroids in which the reverse appeared to be true according to a large series [131]. Sporadic and familial forms of primary parathyroid hyperplasia show indistinguishable histological features and the term "chief cell nodular" or "nodular" hyperplasia appears unsatisfactory, as both diffuse hyperplastic changes and oxyphil cells may occur. The nodules can be single or multiple, are more frequent in larger glands, and vary widely in size and cytoarchitectural pattern (Fig. 23). These may be demarcated by a fibrous stroma or show an abrupt cytoarchitectural contrast with adjacent hyperplastic tissue. In many instances no clear-cut distinction between nodular and diffuse hyperplastic areas can be made. Individual glands may be diffusely hyperplastic and well delineated by the parathyroid capsule resembling an adenoma, whereas diffuse and nodular hyperplastic areas may occasion-

ally show fatty tissue resembling a normal parathyroid [4,85, 86,131,133] (Fig. 23). Nodular and diffuse areas can be composed of classic chief cells, small dark chief cells, chief cells with optically clear cytoplasm, oxyphil cells, or an admixture. These can be arranged in solid, peripheral palisading, trabecular, cribriform, glandular, papillary, and microcystic patterns, or an admixture (Fig. 23). Nuclear pleomorphism and cell multinucleation may occur [85,131,133]. Glandular structures may look empty, show colloidlike material, or contain dense eosinophilic amyloid [23,25] that often immunoreacts with PTH [131]. Hyperplastic parathyroid tissue with a high mitotic rate has been noted [106], particularly in cases presenting with hyperparathyroid crisis [107]. Stromal tissue may show mild lymphoid infiltrates and degenerate changes [131] that may be prominent after fine needle biopsy or therapeutic ethanol injection [104].

Chief cells and oxyphil cells are argyrophilic and show a polymorphic pattern of expression for PTH and PTH mRNA [21, 26,131]. Ultrastructurally, hyperplastic cells show features of parathyroid chief cell differentiation such as membrane-bound secretory granules, while oxyphil cells are characterized by abundant cytoplasmic mitochondria [20,108,133].

Differential diagnoses of primary nodular hyperplasia include nodular hyperplasia from secondary and tertiary hyperparathyroidism which should be differentiated on clinical grounds owing to their similar morphological features. Notwithstanding this, secondary and tertiary hyperparathyroidism shows parathyroids with more prominent internodular diffuse hyperplastic changes than primary chief cell hyperplasia. Parathyroid adenoma usually presents as a solitary lesion as does parathyroid carcinoma, which, in addition, shows evidence of invasion or metastasis. Two or more adenomas involving one or more parathyroid glands resembling primary nodular hyperplasia may occur; thus, antecedents of neck irradiation or a genetically determined defect should be investigated. Differential diagnosis with other neck lesions that may occur in the parathyroid region (e.g., thyroid tumors and paraganglioma) may need the usage of immunocytochemistry.

PRIMARY WATER-CLEAR CELL HYPERPLASIA Primary water-clear cell hyperplasia causes primary hyperparathyroidism in fewer than 5% of cases and shows a wide age range of presentation with the highest frequency in the fifth and sixth decades. Although the suggestion that water-clear cells are derived from chief cells is plausible [108], it is difficult to accept water-clear cell hyperplasia as a variant of primary nodular hyperplasia because it shows distinct morphological features, occurs more frequently in males, and is not known to be familial or occur in association with diseases of other endocrine glands. It shows a strong association with blood group O and for unknown reasons its incidence has declined with time [73,85,86,134]. The patients usually present marked clinical symptoms of hyperparathyroidism with high frequency of urolithiasis and even hyperparathyroid crisis. Occasional asymptomatic cases have been described. All glands are usually enlarged [4,85,86,134]. The disease has been described in supernumerary glands [135] and as affecting two or three glands where no others could be found [86,119]. Here the possibility of parathyroid gland fusion during the hyperplastic process [73] or overlooking an involved gland during surgery could not be excluded [134].

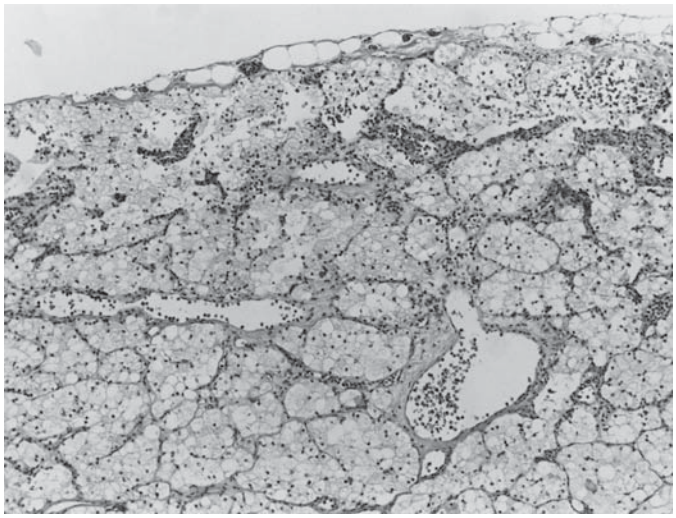

Figure 24 Water-clear cell hyperplasia. Diffuse enlargement of a parathyroid gland displaying trabecular and nesting component of cells with abundant clear vacuolated cytoplasma (H&E, original magnification ×80).

The upper glands are often larger than the lower with a combined weight of up to 125 g but usually ranging between 1 and 50 g [85,86,134,135]. The glands are chocolate brown in color, lobulated to pseudopodal in shape, and the cut surface is uniform often showing discrete cysts [4,73]. Histologically, there is diffuse hyperplasia of cells with abundant well demarcated and granular to finely vacuolated clear cytoplasm with regular nuclei. The cells can be arranged in solid, trabecular, glandular, microcystic, and papillary patterns, or an admixture (Fig. 24). Glandular structures are lined by tall cells with basal nuclei and often contain colloidlike material. A minor classic chief cell component may occur and, occasionally, a rim of normal parathyroid tissue can be found around hyperplastic tissue resembling an adenoma [73,85,86,119]. At ultrastructure, water-clear cells show characteristic 0.2–2 mm membrane-limited vacuoles identifiable on semithin sections as well. Differentiating features of chief cells such as secretory granules are also present [20,108].

The main differential diagnoses is with water-clear cell adenoma where only one gland is affected. Hyperplasia from secondary and tertiary hyperparathyroidism usually show nodular changes; oxyphil cells and the chief cells lack typical water-clear cell histological and ultrastructural features.

LIPOHYPERPLASIA Parathyroid lipohyperplasia is rare and has been described in sporadic form [136] and associated with familial benign hypocalciuric hypercalcemia, a disease linked to inactivating mutations of the calcium sensing receptor gene [93]. All four parathyroid glands are usually enlarged, yellow-tan in color, most of them show an individual weight of 100–200 mg, and have an admixture of fat and neoplastic chief and oxyphil cells. The amount of fat is variable from case to case as well as from gland to gland, with a proportion of fat similar to that seen in normal parathyroids [93,136]. Differential diagnoses include the "solitary" lipoadenoma and primary, secondary, and tertiary parathyroid hyperplasia where the fat cell component is absent or scarce [131].

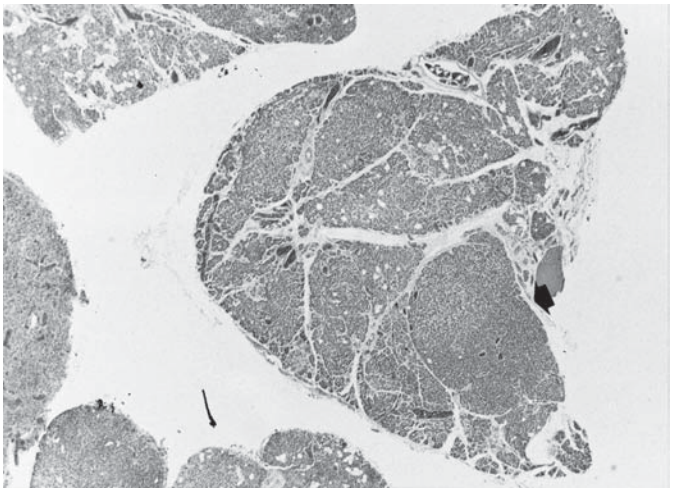

Figure 25 Parathyroid gland from autopsy showing diffuse chief cell hyperplasia with a tendency to focal micronodule formation (arrow) in a patient with secondary hyperparathyroidism due to chronic renal failure (H&E, original magnification ×13).

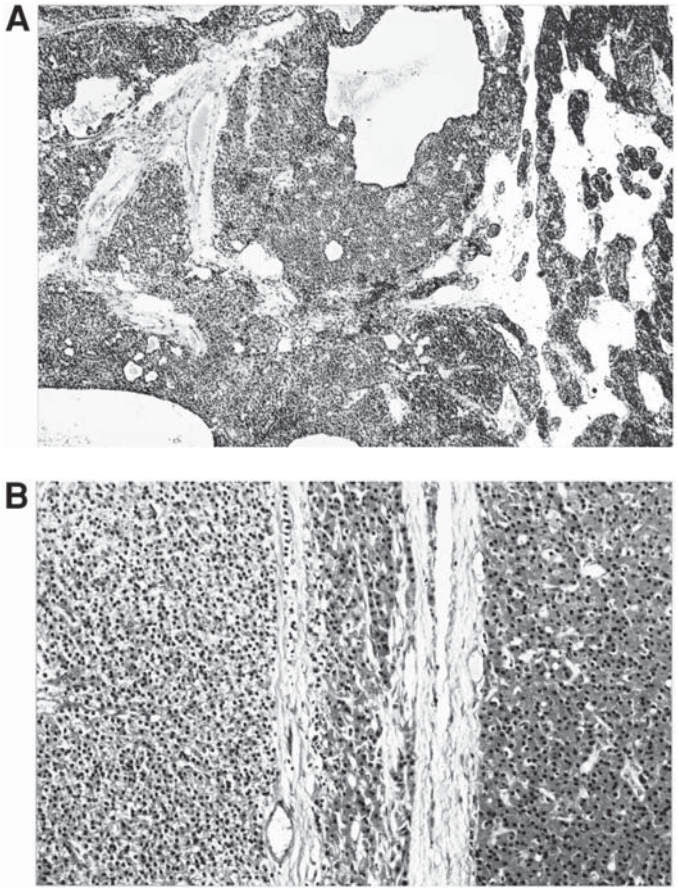

Figure 26 (**A**) Parathyroid gland from a uremic patient with tertiary hyperparathyroidism showing a) diffuse and nodular chief cell hyperplasia adjacent to normal looking parathyroid tissue (H&E, original magnification ×20), and (**B**) nodules composed of chief cells and oxyphil cells arranged in a mainly solid growth pattern (H&E, original magnification ×180).

SECONDARY AND TERTIARY HYPERPARATHYROIDISM

Secondary hyperparathyroidism refers to an adaptive increase of serum PTH due to hypocalcemia that reverts to normal if the clinical derangement is brought under control, while in tertiary hyperparathyroidism one or more autonomous tumors (nodules) with nonsuppressible PTH secretion develop as a result of long-standing secondary hyperparathyroidism. Both disorders occur as a consequence of chronic renal failure and, more rarely, dietary deficiency of vitamin D and calcium (e.g., malabsorption syndromes), tissue resistance to vitamin D, and severe hypomagnesemia [5,137,138]. Secondary hyperparathyroidism was found to be highly prevalent in the elderly in relation to decline in renal function with age, and poor calcium and vitamin D intakes [139].

The classical microscopical picture of secondary hyperparathyroidism is that of a diffuse hyperplasia of chief cells often showing a vacuolated cytoplasm arranged in solid, trabecular, and, less frequently, glandular structures. The relative fat content is usually low or absent and the parenchymal cell mass is increased (Fig. 25). Oxyphil cells increase in frequency with increasing severity of renal failure and, as well as chief cells, may form poorly to well-defined nodules, making the picture indistinguishable from glands with primary nodular and tertiary hyperparathyroidism [30,137,140,141]. Nuclear DNA content is increased in the hyperplastic cells and a hyperdiploid pattern is often present in the lower parathyroid glands, which were reported to have a larger parenchymal cell mass than the upper [142].

In tertiary hyperparathyroidism the superior parathyroid glands were reported to be larger than the inferior with a combined weight of 3.1–12.43 g. Morphological and immunocytochemical features may be indistinguishable from primary chief cell hyperplasia and nodular suppressible secondary hyperplasia (Fig. 26), except that the parathyroid glands in tertiary hyperparathyroidism are usually larger and degenerate changes more common [141,143]. Large eosinophilic ganglionlike cells have been described in hyperplastic parathyroid tissue from a uremic patient with tuberous sclerosis [144]. PTH mRNA is highly expressed in both chief cell and oxyphil cell nodules [64] while normal looking background parathyroid tissue usually shows immunocytochemically negative or very faint PTH storage suggesting suppression [143]. At ultrastructure, hyperplastic chief cells from both secondary and tertiary hyperparathyroidism often show a reduced number of secretory granules, suggesting rapid hormone release [137]. Oxyphil cells are characterized by abundant cytoplasmic mitochondria [30,137,140]. A higher recurrence rate of hyperparathyroidism after parathyroidectomy can be predicted in patients whose parathyroids show a nodular growth pattern and elevated proliferative rate as shown by Ki-67 immunostaining [67].

Morphological differentiation between primary, secondary, and tertiary parathyroid hyperplasia may not always be possible without knowledge of clinical data, especially in glands incidentally found on postmortem examination or removal during thyroidectomy or laryngectomy. Secondary and tertiary hyperparathyroidism show parathyroids with more prominent

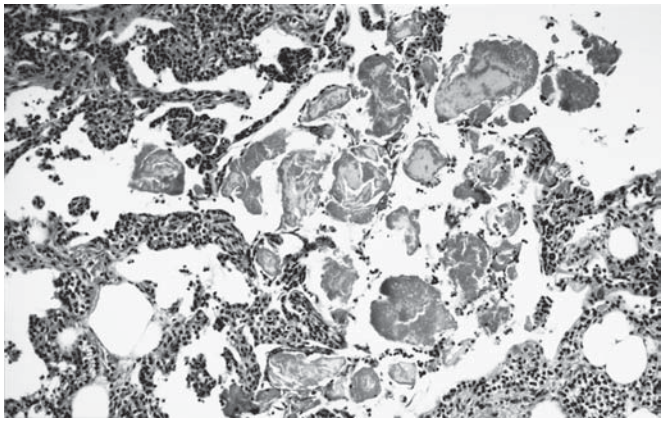

Figure 27 Stromal amyloid deposits in a parathyroid gland from a patient with rheumatoid arthritis (H&E, original magnification ×80).

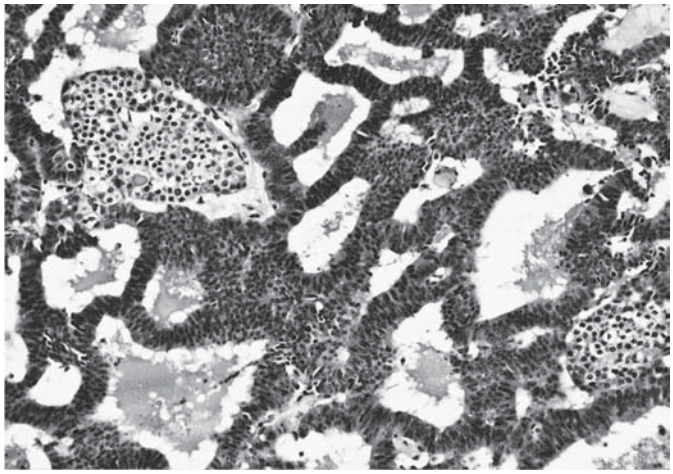

Figure 28 Metastatic bronchial carcinoid in hyperplastic parathyroid tissue from a 50-yr-old male with MEN 1 and hyperparathyroidism (H&E, original magnification ×80).

internodular diffuse hyperplastic changes than primary chief cell hyperplasia. Primary water-clear cell hyperplasia reveals distinct histological and ultrastructural features but neither nodular changes nor oxyphil cells.

HYPOPARATHYROIDISM, PARATHYROIDITIS, AND MISCELLANEOUS DISORDERS

Hypoparathyrodism is diagnosed on the basis of measurement of serum calcium and PTH. It can cause hypocalcemia, paresthesias, muscle spasms (tetany), and seizures when it occurs rapidly, or presents with visual symptoms due to cataracts in chronic states of hypocalcemia [5]. The most common cause of hypoparathyroidism is related to medical treatment following removal of the parathyroid glands during thyroidectomy and laryngectomy. Less frequently, hypoparathyroidism may follow infarction of a parathyroid gland remnant after subtotal parathyroidectomy [61] or necrosis and fibrosis of hyperplastic parathyroid glands after percutaneous therapeutic ethanol injection [104].

Amyloidosis involving all four parathyroid glands may lead to hypoparathyroidism, which is rarely the major clinical problem. Amyloid proteins AL (amyloid light chain) and AA (amyloid associated) occur probably more frequently than thought in the parathyroid glands as these are not usually routinely examined at postmortem. AL amyloidosis is associated with multiple myeloma and other monoclonal B-cell proliferations. AA amyloidosis is commonly related to long-standing illnesses such as chronic infections, rheumatoid arthritis, and Crohn's disease. Compensatory hyperplasia admixed with amyloid deposits can be noted in parathyroids from patients with chronic renal failure due to generalized amyloidosis [145]. Congophilic green birefringent amyloid deposits are observed around the walls of arteries, arterioles, capillaries, and, less frequently, within stroma tissue (Fig. 27). The affinity of amyloid for Congo red is reduced after potassium permanganate treatment in AA amyloid, but the staining persists in primary amyloidosis and in amyloid from parathyroid follicles as seen in normal, hyperplastic, and neoplastic parathyroid glands [23,145]. Immunocytochemistry for specific amyloid proteins may also help in their distinction.

Pseudohypoparathyroidism is manifested by hypocalcemia related to PTH tissue unresponsiveness at the receptor level. This is one feature of Albright's hereditary osteodystrophy, an autosomal dominant trait characterized by short stature, obesity, subcutaneous ossification, focal skeletal defects, and rounded faces. No histological abnormalities were detected in parathyroid glands from patients with this disorder [146].

Progressive systemic sclerosis may produce markedly diffuse parathyroid gland fibrosis and lead to hypoparathyroidism [147]. In Riedel's thyroiditis the parathyroid gland may also be involved by invasive fibrous tissue [148].

Secondary parathyroid malignancies affecting at least one parathyroid gland were found to occur in up to 12% of autopsies from patients with cancer, including breast carcinoma, leukemia/lymphoma, and malignant melanoma (Fig. 28). Hypoparathyroidism occurred when at least 70% of parathyroid tissue from all glands were involved [149].

Chronic parathyroiditis, analogous to Hashimoto's thyroiditis, occurs in the autoimmune polyglandular syndrome type 1 (Addison's disease, mucocutaneous moniliasis, and hypoparathyroidism), and also as an assumed autoimmune sporadic condition that may present with or without overt symptoms of hyperparathyroidism. In the latter, diffuse to nodular enlargement of at least two parathyroid glands has been described. Histologically, there are destructive patchy to diffuse polyclonal infiltrates of plasma cells and lymphocytes admixed with bands of fibrous tissue surrounding residual islands of chief and/or oxyphil cells that may show nuclear pleomorphism (Fig. 29). Lymphoid follicles forming germinal centers may occur. Admixed areas of diffuse or nodular parathyroid cell hyperplasia can be present [74,150–152]. Mild lymphoid infiltrates have been reported to occur in about 10% of normal parathyroid glands at autopsy [152] and, occasionally, in parathyroid hyperplasia [131, 143]. Parathyroid adenomas replaced by destructive lymphocytic infiltrates resembling chronic parathyroiditis have been described [153].

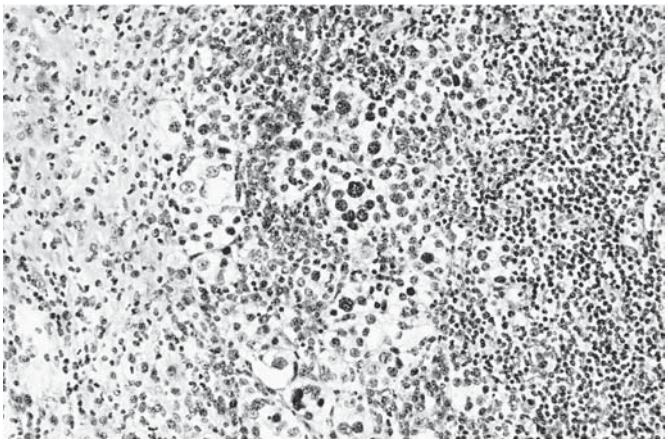

Figure 29 Lymphoid infiltrates and fibrosis admixed with surviving nests of chief cells sometimes showing nuclear pleomorphism from a 74-yr-old man presenting with hyperparathyroidism (H&E, original magnification ×360).

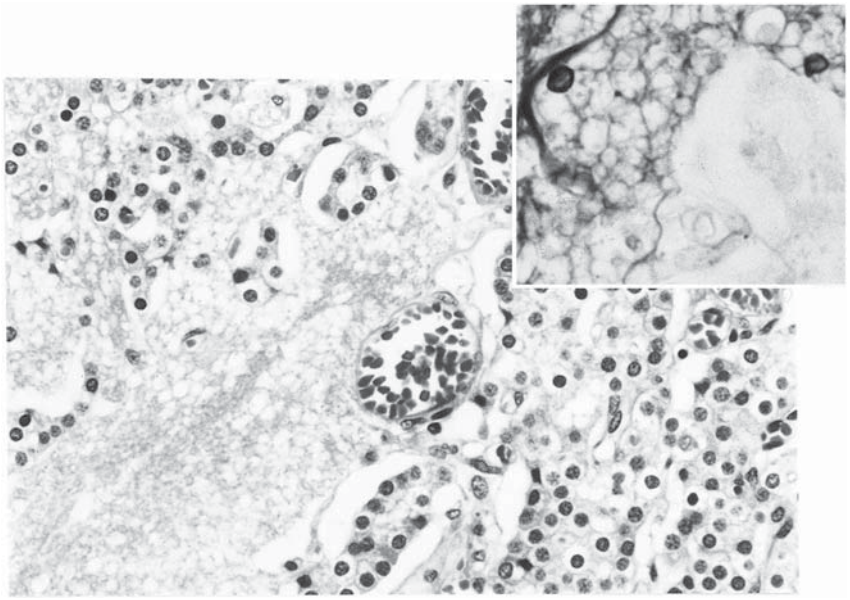

Figure 30 Parathyroid tissue from a 33-yr-old man with AIDS replaced by pink foamy material (H&E, original magnification ×360) containing *Pneumocystis carinii* (*inset*: Groccot silver stain, original magnification ×360) (Courtesy of Professor Sebastian Lucas.)

Infectious parathyroiditis caused by Cytomegalovirus and *Pneumocystis carinii* have been described in immunocompromised patients due to corticosteroid therapy and AIDS [154, 155]. Cytomegalovirus presents histologically as characteristic large purple intranuclear inclusions surrounded by a clear halo in the chief cells. The viral inclusions can be readilly identified by immunocytochemistry or *in situ* hybridization. In *P. carinii* infection the parathyroid parenchyma is replaced in various extents by eosinophilic foamy material containing cell debris and pale pinkish cup-shaped 4–6-μm parasitic cysts which are more easily identified with Giemsa, toluidine blue, or silver stains (Fig. 30), and by immunocytochemistry. Disseminated noncaseating granulomas produced by *Mycobacterium tuberculosis* have been described in a parathyroid adenoma [156], and should be distinguished from sarcoidal granulomata involving the parathyroid gland [79].

Cystinosis shows widespread intracellular deposition of cystine. Brick-shaped cystine crystals were described to occur in the stroma of the parathyroid glands. The crystals are soluble in common fixatives but well preserved in frozen section or absolute alcohol fixation and show birefringence after polarization. In cystinosis there is no apparent clinical parathyroid dysfunction although hypothyroidism may occur [157]. Hypoparathyroidism has also been described following ^{131}I therapy for hyperthyroidism [158], in association with longstanding iron storage disease [159] and in Wilson's disease [160].

Glycogen storage disease type II (Pompe's disease) shows intralysosomal glycogen storage in multiple organs, including

the parathyroid glands. Chief cells show marked cytoplasmic PAS positivity and electron microscopy discloses abundant glycogen granules dispersed free in the cell cytoplasm and also bound by lysosomal membranes. Glycogen may be found within the nucleus [161].

Parathyroid peliosis [162], Langerhans cell histiocytosis [164], and angiomatous proliferations [163] have been described.

INTRAOPERATIVE DIAGNOSIS OF PARATHYROID DISEASE

The major task of the pathologist intraoperatively is to confirm the presence of parathyroid tissue for surgical decision making; this can be done either with frozen section or cytological touch preparation. Experience is required for cytological diagnosis that is quick and cost effective. This method may be useful particularly for tiny tissues that are difficult to cut such as a small normal parathyroid gland, small biopsy from a parathyroid lesion, parathyromatosis, and obvious multiple gland enlargement as seen in tertiary hyperparathyroidism, for example. Cytological touch preparations can also help in identifying parathyroid parenchymal cells in lipoadenoma or lipohyperplasia with a large fatty tissue component impossible to be thoroughly sampled, and where differential diagnosis with lipoma of the neck may arise [143].

The second step is to describe the lesion and, when appropriate, confirm normal parathyroid tissue. Very rarely, distinct invasion by parathyroid carcinoma can be seen on frozen section. The most common situation in parathyroid surgery is that a single enlarged parathyroid gland is excised together with a second normal parathyroid gland, findings that are sufficient to stop surgical search for more parathyroids, as the possibility of a parathyroid adenoma is high in this situation. Differential diagnosis rarely in any case influences the intraoperative surgical decisions which are based on the number and size of confirmed parathyroid glands found. It is not recommended to make a definitive diagnosis unless obvious.

Parathyroid lesions may show a dominant glandular architectural component that may be difficult to differentiate from thyroid tissue. This situation may arise especially when the parathyroid lesion occurs ectopically within the thyroid, when a parathyroid carcinoma invades the thyroid, and when either accessory thyroid tissue found detached from the main gland or a metastatic lymph node completely replaced by a follicular cell carcinoma are removed and suspected by the surgeon to be parathyroid. The use of polarized light during frozen section may help to identify birefringent oxalate crystals that are sometimes present in thyroid colloid but do not occur in parathyroid glandular structures [58,59], or to identify birefringent amyloid fibers that may occur within lumina from parathyroid glandular structures but are not present in thyroid colloid [58]. When no parathyroid gland enlargement is found during intraoperative diagnosis in the setting of primary hyperparathyroidism the surgeon will normally look for an ectopic enlarged parathyroid gland, especially in the thyroid, thymus, or carotid sheath. If the search for ectopic tissue failed surgery will probably be stopped and further decision deferred. Under these conditions the possibility of a parathyroid microadenoma should be considered and multiple sectioning may be required to identify it in an otherwise normal parathyroid gland.

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8 Recent Developments in the Molecular Biology of the Parathyroid

VÂNIA NOSÉ, MD, PHD AND ASHRAF KHAN, MD, FRCPATH

MOLECULAR GENETICS OF THE PARATHYROID CELLS

HISTORICAL OVERVIEW Virchow first described the parathyroid glands in 1863. Ivar Sandstrom published detailed descriptions in 1880. In recent years, major advances have occurred in the study of the molecular and genetic aspects of these glands.

Askanazy described the first neoplasm of the parathyroid glands in 1903, found at an autopsy of a patient with von Recklinghausen's disease. In 1925, Mandl in Vienna removed the first parathyroid tumor from a patient with the same disease.

In 1926, the first surgery for hyperparathyroidism in the United States was performed at Massachusetts General Hospital, in a patient found at another surgery to have a mediastinal parathyroid adenoma. In 1934, the first case of parathyroid carcinoma was reported by Hall and Chaffin. The first series of histopathology findings in hyperparathyroidism was from Benjamin Castleman and T.B. Mallory, in 1935 [1,2].

The significance and function of the parathyroid began to be understood by F. Albright in 1948 during experimental studies of tetanus associated with removal of the glands. Albright found the relationship between tetanus, hypocalcemia, and the characterization of the parathyroid hormone [3].

Knowledge of the involvement of the parathyroid in diverse syndromes has been expanded since the first report of Wermer syndrome in 1954, and of Zollinger–Ellison syndrome in the following year [4,5]. That these two syndromes are in fact the same multiple endocrine lesion was subsequently recognized by Lulu in 1968 [6]. The preferred designation for this phenomenon is multiple endocrine neoplasia type 1 (MEN 1).

The amino acid sequence of the parathyroid hormone (PTH) was first determined in 1997. Nucleotide sequence of the gene followed in 1990 [7,8].

The *PTH* gene was assigned to 11p15, along with insulin, H-*ras*, and β-hemoglobin, by *in situ* hybridization [9].

A parathyroid hormone–parathyroid hormone–related peptide (PTH–PTHrP) receptor was then reported. This receptor, which binds PTH and PTHrP, with seven potential membrane-spanning domains, was subsequently cloned. It showed homology with the calcitonin receptor and no homology with other G-protein-linked receptors, indicating that these receptors represent a new family [10,11].

The *PTH-R* gene was mapped in 1994 to chromosome 3p22–p21.1 [12] and redefined the vicinity of the 3p21.3–p21.2 [13].

A chromosomal breakpoint of chronic lymphocytic leukemia cells of the B-cell type with t(11;14)(q13;q32) was cloned in 1984; this gene was designated *BCL1* [14]. Two parathyroid adenomas bearing clonal restriction fragment abnormalities involving the parathyroid hormone locus on 11p and reciprocal rearrangement with the 11q13 region were reported in 1989 [15].

A somatic mutation describing the *PRAD1* gene under the promoter of the parathyroid hormone gene was reported in some parathyroid adenomas in 1991 [16]. In 1994, a conclusion that the *BCL1* gene is the same as *PRAD1*, known today as *cyclin D1*, was reported [17].

A parathyroid cell Ca^{2+}-sensing receptor (*CaR*) cDNA encoding a 120-kDa polypeptide, present also in the kidney, was identified in 1993 [18].

An association with mutations in the human *CaR* gene was reported in 1993 in both familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism [19].

The *CaR* gene was assigned to 3q13.3–q21 by fluorescence *in situ* hybridization (FISH). This was confirmed by somatic cell hybrid analysis [20].

Baron et al. (1996) identified two families with autosomal dominant hypoparathyroidism with heterozygous mutations in the *CaR* gene, and identified a *de novo CaR* missense in an infant with severe hypoparathyroidism [21].

Direct sequencing of the *CaR* gene revealed no mutations in 20 sporadic parathyroid adenomas [22].

Hendy et al. [23] reviewed the CaR mutations in familial hypocalciuric hypercalcemia, neonatal severe hyperparathyroidism, and autosomal dominant hypocalcemia [23].

In 1988, the *MEN1* locus was mapped to chromosome 11 by demonstrating linkage to a DNA probe derived from the *PYGM* locus. The *PYGM* locus has in turn been mapped to 11q13–qter [24]. Two groups have demonstrated loss of heterozygosity for chromosome 11 alleles in parathyroid tumors from patients with MEN 1 [25,26]. The European Consortium on MEN 1 in

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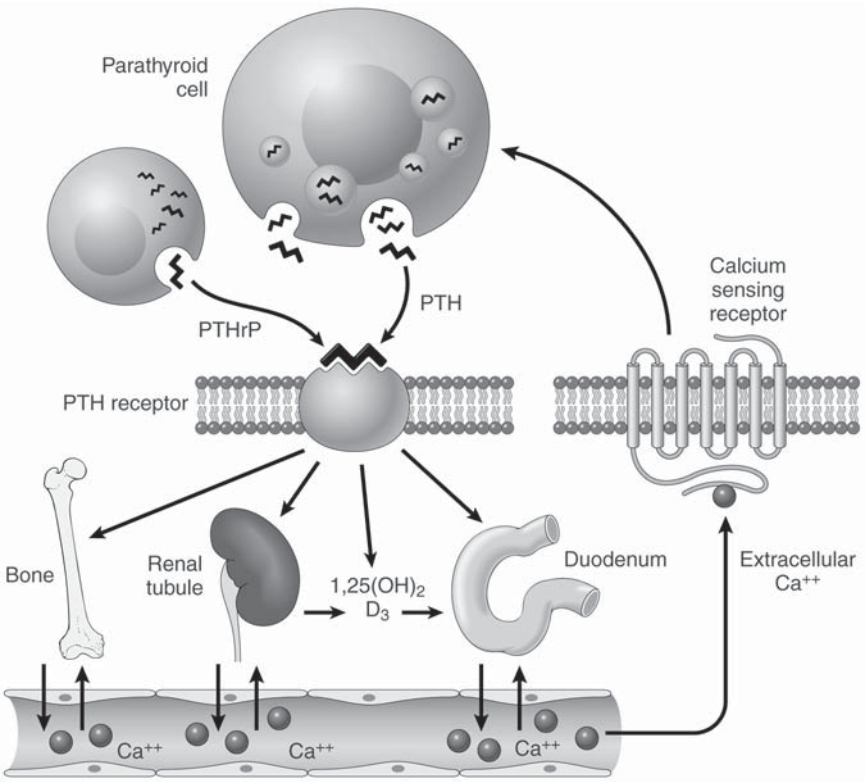

Figure 1 Calcium homeostasis: the parathyroid axis. Activation of the calcium receptor at the parathyroid cell surface leads to increased intracellular calcium, which inhibits PTH secretion. In response to hypocalcemia, there is increase in bone resorption and renal calcium reabsorption, with subsequent increase in calcium levels. $1,25(OH)_2D_3$ increases serum calcium levels by stimulation of intestinal calcium absorption and inhibition of PTH secretion. PTH and PTHrP act through the same receptor (PTH receptor).

1997 constructed a sequence-ready contig and described three gene clusters, including the central cluster that contains the *MEN1* gene [27]. An NIH group identified several *MEN1* candidate genes in a previously identified minimal interval on 11q13 [28]. The protein Menin, a product of the MEN1 gene, was reported in 1998 to be a nuclear protein [29].

THE PARATHYROID AXIS The four parathyroid glands regulate serum calcium concentration, calcium homeostasis, calcitonin, bone metabolism, and vitamin D through the secretion of parathyroid hormone. In return, serum calcium concentrations regulate PTH secretion.

A complex homeostatic system maintains the serum calcium concentration. The fluctuations in extracellular calcium concentration are detected by the parathyroid cells, which respond with rapid changes in the secretion of PTH. It has been demonstrated that this capacity is mostly mediated via the CaR expressed in the parathyroid cells, C cells of the thyroid, and by the renal cells. In response to decreased serum calcium concentration, the CaR on the surface of the parathyroid cells stimulates the release of parathyroid hormone from the chief cells within seconds. This is followed by an increase in PTH mRNA within hours of the detection of hypocalcemia. Conversely, hypercalcemia inhibits the release of PTH by activating the calcium receptor of the parathyroid cells. High serum phosphate levels also increase PTH secretion independently of changes in serum calcium or vitamin D levels. The effect of phosphate on the parathyroid is mediated by phospholipase A_2. Parathyroid cell proliferation is increased by chronic hyperphosphatemia and decreased by hypophosphatemia. The parathyroid cells regulate the synthesis, storage, and release of PTH and PTHrP. Both PTH and PTHrP act through the same receptor, *PTH1* receptor, in the renal tubules, bone, and duodenum, to absorb and excrete calcium and maintain calcium homeostasis (Fig. 1).

PTH PTH is synthesized, stored, and secreted mostly by the chief cells of the parathyroid. Initially, it is synthesized as a 115-amino-acid-containing precursor, a preproparathyroid hormone, within the cytoplasm of the parathyroid cells. When the hormone enters the endoplasmic reticulum, a 25-amino-acid-containing N-terminal fragment is removed. The intermediary protein, the proparathyroid hormone, is transported to the Golgi apparatus. There, cleavage of the N-terminus segment occurs, converting the proparathyroid hormone to parathyroid hormone. Intact PTH is an 84-amino-acid polypeptide with a molecular weight of 9600. It is stored in the secretory granules in association with other proteins, mostly chromogranin A. PTH is degraded in the kidney and liver.

PTH secretion occurs in response to low calcium concentrations. Conversely, high concentrations of calcium inhibit PTH secretion. On the renal tubules, PTH binds to PTH receptors and stimulates the reabsorption of calcium at distal tubules and at the ascending loop of Henle. It inhibits reabsorption of phosphate and bicarbonate at the proximal tubules. This results in increased serum calcium and decreased serum phosphate and

bicarbonate levels. PTH also stimulates the conversion of 25-hydroxycholecalciferol ($25OHD_3$) to the active form of vitamin D (1,25-dihydroxycholecalciferol). In bone, PTH releases calcium and phosphate into the circulation by bone resorption.

By *in situ* hybridization, the human *PTH* gene was mapped to the 11p15 chromosomal band closely linked to the β-hemoglobin gene, the Harvey-*ras* 1 proto-oncogene, and the insulin gene. All of these genes are localized at 11p15, a region of one chromosomal band that appears to comprise a genetic distance of more than 20 cM [9].

PTHrP PTHrP is a homolog of PTH, but is not a true hormone. It shows marked N-terminal homology to PTH. This N-terminal end is actively involved in calcium regulation. Serum calcium levels do not regulate the secretion of the PTHrP. PTH and PTHrP are calciotropic hormones interacting with a shared seven-transmembrane domain G-protein-coupled receptor, which is located predominantly in bone and kidney. PTHrP, with a molecular mass of approx 17kDa, is synthesized in a greater variety of tissues than PTH. Examples of these are cartilage, parathyroid cells, keratinocytes, smooth muscle cells, placenta, and lactating breast tissue.

A phage containing the gene encoding human preproparathyroid hormone was isolated in 1983 [30]. The gene is approx 4200 basepairs long and is located at 12p.

PTHr The *PTHr* gene encodes a receptor for both PTH and PTHrP (Fig. 1). PTHr is a member of a family of G-protein-coupled receptors that includes receptors for secretin, growth hormone-releasing hormone, vasoactive intestinal polypeptide, type 1, gastric-inhibitory polypeptide, glucagon-like peptide 1, glucagon, corticotrophin-releasing factor, and pituitary adenylate cyclase activating peptide 1 [10]. The *PTHr* gene is mapped to chromosome 3 [12]. By isotopic *in situ* hybridization, the chromosomal assignment was defined to 3p22-p21.1.

Jobert et al. demonstrated that the mutational inactivation of PTH receptors was responsible for a genetic disorder characterized by advanced endochondral bone maturation (Blomstrand chondrodysplasia) [31]. Enchondromas can occur as solitary lesions or as multiple lesions, as in enchondromatosis. A mutant PTH receptor in two of six patients with Ollier disease was identified [32].

CaR A putative CaR cDNA was identified in the bovine parathyroid cell. This cDNA encoded a predicted 120-kDa polypeptide with an extracellular domain [18]. Cloning from different tissues in various species followed the cloning of the bovine CaR. CaR, whose gene resides on chromosome 3q13.3–21, is a plasma membrane G-protein-coupled receptor that is expressed in the PTH-producing parathyroid chief cells, renal tubular cells, C cells of the thyroid, bone, and cartilaginous cells. CaR is a heptahelical molecule, similar to other hormone receptors (Figs. 1 and 2). It plays an essential role in maintaining mineral ion homeostasis, due to its special ability to recognize changes in calcium concentration.

The secretion of PTH in response to hypocalcemia is mediated through the CaR. It is expressed on the parathyroid cell surface and senses fluctuations in the concentration of extracellular calcium [33,34]. In response to hypocalcemia, PTH elevates serum calcium through enhanced bone resorption and renal calcium reabsorption (Fig. 1).

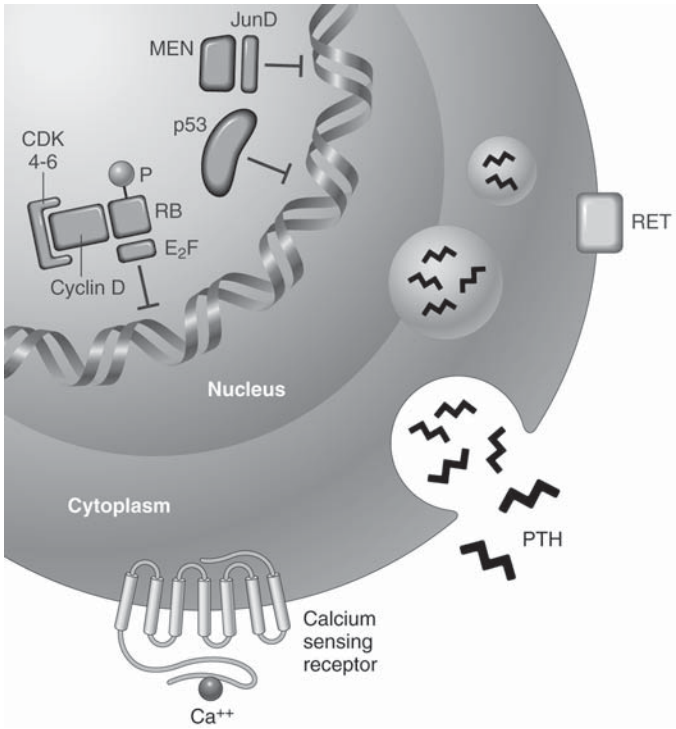

Figure 2 Parathyroid proliferation, gene expression, and secretion. A low serum calcium and/or high serum phosphate lead to a decreased activation of the CaR, with increased PTH secretion. Inactivating mutations in the tumor suppressor-encoded proteins Menin, p53, Rb, and CaR and activating mutations of the protooncoproteins cyclin D1 and RET contribute to parathyroid tumor formation. The exact mechanisms by which these proteins contribute to tumor development is still to be determined.

Inactivating mutation in the CaR of the parathyroid glands and the kidneys can cause familial hypocalciuric hypercalcemia, neonatal severe hyperparathyroidism, and autosomal dominant hypocalcemia. These mutations render the receptor insensitive to calcium, leading to retention of urinary calcium and changes in the calcium–PTH curve. No mutations of the *CaR* gene were found in sporadic parathyroid adenomas and in uremic hyperparathyroidism, but the expression of its protein is reduced on the surface of parathyroid cells in these diseases, probably contributing to the increase in PTH secretion.

VITAMIN D Vitamin D is a fat-soluble sterol, absorbed as ergocalciferol in the intestine or synthesized in the skin after ultraviolet light converts 7-dehydrocholesterol to cholecalciferol. The active vitamin D acts on the gut to promote the absorption of dietary calcium, and on the skeleton to facilitate the action of PTH on bone resorption. Vitamin D is an important factor in calcium homeostasis. Active vitamin D, 1,25-dihydroxycholecalciferol, increases calcium levels by stimulation of absorption of intestinal calcium, and by a negative feedback mechanism inhibiting PTH secretion (Fig. 1). The synthesis of osteocalcin, the most abundant protein in bone, is induced by calcitriol, the active hormonal form of vitamin D [35–37].

Vitamin D and its metabolites, acting through vitamin D receptors, decreases the levels of PTH mRNA.

Metabolites of vitamin D, as 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D, directly inhibit the mass of parathyroid

cells. Acting by itself, hypocalcemia stimulates the growth of parathyroid cells independent of the opponent action of vitamin D metabolites. Alterations in these processes cause hyperparathyroidism or hypoparathyroidism.

VITAMIN D RECEPTOR Baker et al. described the cloning and characterization of cDNAs encoding the human vitamin D receptor [38]. The vitamin D3 receptors are intracellular polypeptides of 50–60 kDa that specifically bind 1,25-dihydroxycholecalciferol. The vitamin D receptor belongs to the steroid-receptor gene family; its sequence and size are similar to those of the thyroid hormone receptor. The human *VDR* gene and its promoter were characterized in 1997 [39]. The *VDR* gene contains 11 exons and spans approx 75 kb.

The *VDR* gene is located on 12q in the human [40]. The gene was assigned to 12q12–q14 by *in situ* hybridization [41].

The *COL2A1* and *VDR* loci, both located on chromosome 12q12, are separated by <740 kb, with *VDR* distal to *COL2A1* [42]. *VDR* polymorphism comprises a risk factor for the development of sporadic primary hyperparathyroidism, mostly in females and individuals developing parathyroid adenoma [43]. In MEN 1 and uremia, hyperparathyroidism was found to be unrelated to *VDR* polymorphism [44,45].

CALCITONIN Human calcitonin contains 32 amino acids and has a molecular weight of 3421. It is a peptide hormone synthesized by the parafollicular C cells of the thyroid. Calcitonin is responsible for decreasing serum calcium, the opposite of the effect of PTH. It is secreted in response to a number of stimuli, the major one being a high calcium concentration. Some other stimulants are high magnesium, glucagon, gastrin, and cholecystokinin. Conversely, a low calcium concentration, dopamine, and α-agonists inhibit calcitonin secretion. High concentrations of calcitonin decrease calcium and phosphate reabsorption from renal tubules and also decrease osteoclastic bone resorption.

The calcitonin gene was assigned to 11p14–qter [46]. The calcitonin gene is alternatively expressed in a tissue-specific fashion producing either the calcium regulatory hormone calcitonin or calcitonin gene-related peptide [47]. The calcitonin 2 gene produces a second calcitonin gene-related peptide, not a second calcitonin. The pseudogene *CALC3* does not encode either peptide. By FISH to prometaphase chromosomes, two-color *in situ* hybridization to interphase nuclei and pulsed field gel electrophoresis analysis, *CALCA*, *CALCB*, and the pseudogene *CALC3* were mapped to 11p15.2–p15.1 [48,49].

A human calcitonin receptor cDNA was cloned from an ovarian small cell carcinoma cell line [47]. The same group cloned and characterized two distinct calcitonin receptor-encoding cDNAs from a giant cell tumor of bone and demonstrated that the *CALCR* gene is located on 7q22. FISH was used to map the *CALCR* gene to 7q21.3 [47,50].

MOLECULAR GENETICS OF HYPERPARATHYROIDISM

HYPERPARATHYROIDISM—INTRODUCTION The parathyroid diseases have been classified according to their metabolic and hormonal status as hypoparathyroidism and hyperparathyroidism.

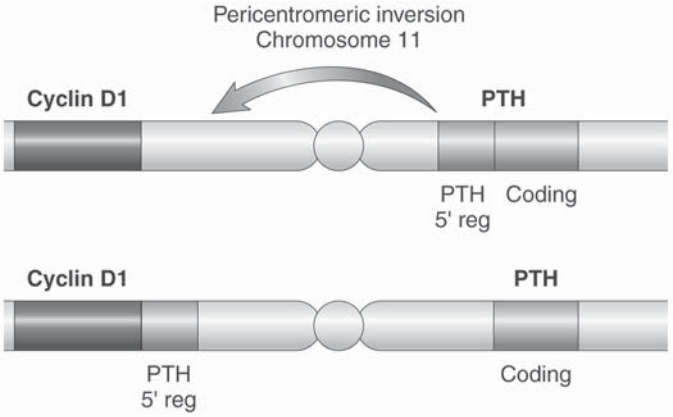

Figure 3 Chromosome 11 *cyclin D1–PTH* rearrangement. A pericentromeric inversion of 11p (*PTH*) and 11q13(*cyclin D1*) places the *cyclin D1* under the regulatory region of the gene encoding PTH, causing overexpression of *cyclin D1*. The rearrangement separates the *PTH* gene's 5' flanking region from its coding exons.

Hyperparathyroidism refers to a status of increased production of PTH, with normal or abnormal serum calcium. It is classified as primary or secondary. Primary hyperparathyroidism is a common endocrinopathy, familial or not in origin, due to hyperplasia, adenoma, or carcinoma.

Most cases of nonfamilial hyperparathyroidism are due to a single adenoma. Multiglandular parathyroid hyperplasia and parathyroid carcinoma are less frequent. Most of the genetic bases of these diseases have yet to be defined.

Some cases of sporadic nonfamilial parathyroid adenomas were found to be associated with genetic abnormalities as a pericentromeric inversion on the chromosome 11 (Fig. 3). Many other chromosomal changes were identified in these sporadic adenomas, some of which are related to genes associated with familial diseases, as mutations and deletions in the *MEN1* gene. Various chromosomal regions, many loci containing oncogenes, tumor suppressor genes, and genes for calcium-sensing receptors are implicated in the development of some of these tumors. Loss of heterozygozity of 11q13, a region containing *cyclin D1*, *MEN 1*, and other important oncogenes and tumor suppressor genes (Fig. 4), is the most frequent finding in parathyroid tumors, both sporadic adenomas and *MEN1*-related parathyroid adenomas [37,51–63].

Abnormalities of the retinoblastoma (*Rb*) and *p53* tumor suppressor genes, involved in tumorigenesis of different tumors, are also found in parathyroid neoplasia. Overexpression of *p53* has been observed in parathyroid adenomas and carcinomas but allelic loss of *Rb* is found to be specific for parathyroid carcinomas [64–70].

The familial form of hyperparathyroidism is found in many autosomal dominant disorders, among them *MEN1*, *MEN2A*, hereditary hyperparathyroidism–jaw tumor syndrome, and familial isolated hyperparathyroidism. Recent genetic information has added to the identification of specific hyperparathyroid diseases, such as neonatal severe hyperparathyroidism, familial hypocalciuric hypercalcemia, autosomal dominant mild hyperparathyroidism, and, more recently, familial hypercalcemia and hypercalciuria.

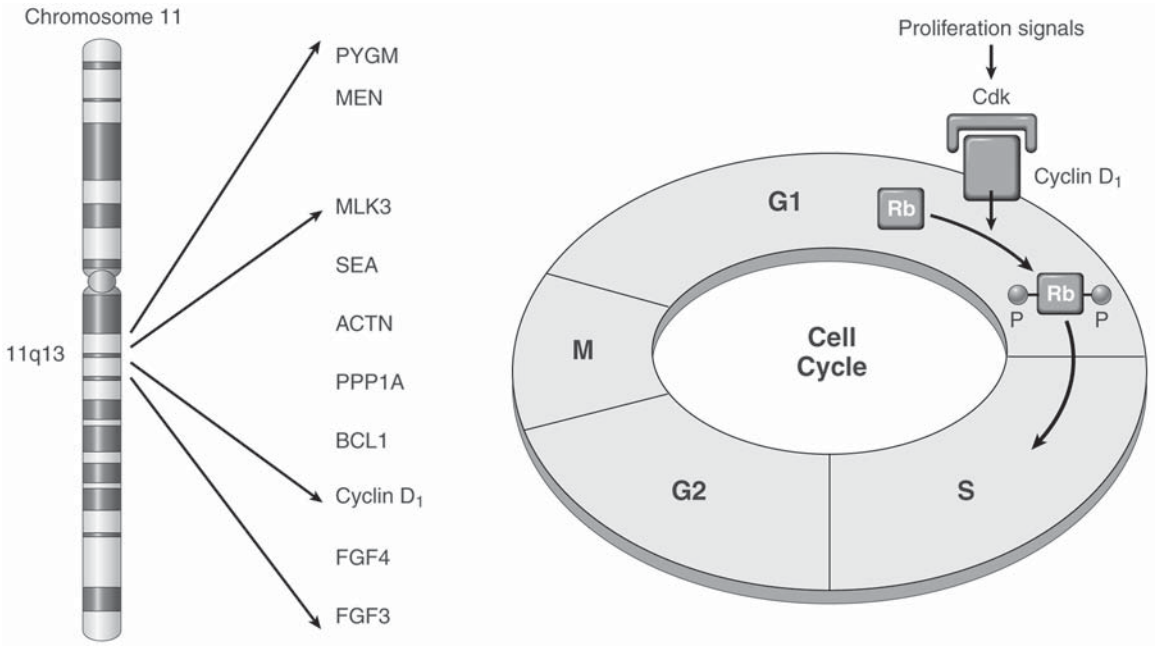

Figure 4 Action of cyclin D1 and Rb in the cell cycle. Cyclin D1 interacts with Cyclin D-dependent kinases 4 and 6 (Cdk) leading to hyperphosphorylation of Rb. The inactive Rb allows the cell to enter the G_1–S phase of the cell cycle.

Secondary hyperparathyroidism due to renal failure, hypocalcemia, and hyperphosphatemia leads to increased PTH synthesis and secretion, and proliferation of the parathyroid cells (Fig. 5). The development of an autonomous gland in patients with secondary hyperparathyroidism is better described as tertiary hyperparathyroidism. These two diseases have overlapping histology and clonality. The mechanisms of monoclonal proliferation in uremic hyperparathyroidism are not well understood. How these stimuli contribute to parathyroid cell hyperplasia and neoplasia are in the process of being clarified. Numerous genetic abnormalities have been confirmed as taking part in uremic hyperparathyroidism, including chromosomal losses in the calcium-sensing gene chromosomal region 3q. The findings of reduction of vitamin D receptors as cause of progression of secondary hyperparathyroidism as well as abnormalities in the mechanism of the vitamin D–vitamin D receptor complex might explain their contribution to parathyroid tumorigenesis [71,72].

Hyperparathyroidism has two major findings: calcium-insensitive hypersecretion of PTH and increased parathyroid cell proliferation. Because of this cell proliferation, the histopathology of hyperparathyroidism itself is inadequate to differentiate between the two disorders. Newer techniques and emerging newer concepts should be incorporated in the actual classification of parathyroid diseases. A well-balanced correlation of gene expression profiling with the morphological findings will complement each other and will help the understanding and future classifications of most endocrine lesions. Recent developments in the study of the molecular genetics of these different types of hyperparathyroidism have enhanced the differentiation between the diseases, and future studies will undoubtedly do the same.

PRIMARY AND SPORADIC Hyperparathyroidism can occur in isolated or familial forms. Sporadic primary hyperparathyroidism is mostly of unknown etiology. It is predominant in women >50 yr old. External irradiation to the neck is a contributing risk factor for these sporadic forms.

Primary hyperparathyroidism is caused by inappropriate secretion of PTH, leading to hypercalcemia. About 85% of cases are caused by a monoclonal adenoma, while multiglandular parathyroid hyperplasia accounts for approx 15–20%, with carcinomas accounting for rare cases. Some cases are part of inherited syndromes (Table 1).

Solitary parathyroid adenomas or multiglandular hyperparathyroidism are monoclonal or oligoclonal proliferations. The underlying genes that develop mutations and are responsible for the hyperparathyroidism are known only in the minority of parathyroid proliferations.

During the last decade, some of the genetic mechanisms related to parathyroid tumorigenesis have been clarified. Familial syndromes have been mapped to deletions of chromosomal regions. The 11q13 region, which harbors the gene for *cyclin D1* and the *MEN1* tumor suppressor gene (Fig. 4), is deleted in a third of sporadic primary hyperparathyroidism. In approximately half of these cases, a somatic mutation of *MEN1* has been identified [73–78]. Inactivation of the *MEN1* gene is an important genetic alteration involved in the development of parathyroid tumors in post-irradiation patients.

Chromosomal rearrangements are described in soft tissue tumors and in leukemia and lymphomas and only few epithelial cell tumors have been correlated with specific chromosomal rearrangements with resulting fusion proteins demonstrated to contribute to the neoplastic process. In parathyroid lesions the pericentromeric inversion of 11p15 and 11q13, placing the

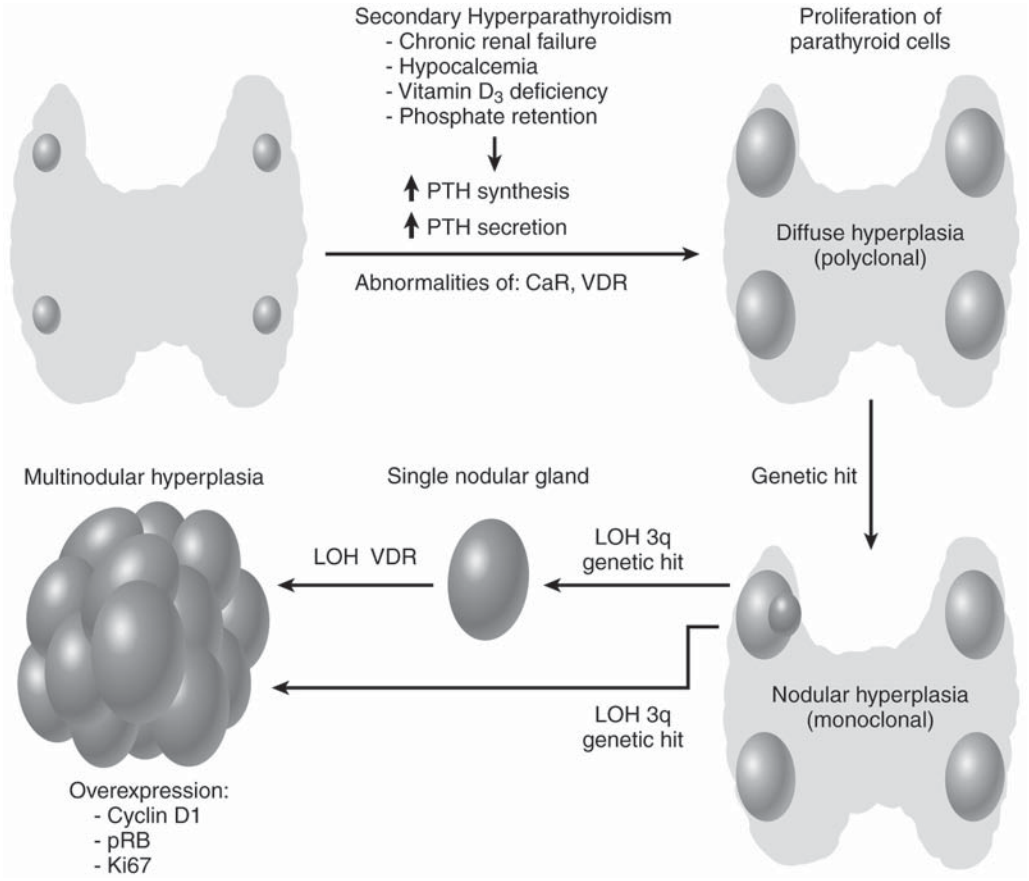

Figure 5 Secondary hyperparathyroidism. Chronic renal failure, hypocalcemia, phosphate retention, and vitamin D deficiency cause diffuse cell proliferation of the parathyroid. The initial parathyroid hyperplasia is polyclonal, and by different genetic hits, it can become monoclonal.

cyclin D1 under the regulatory region of the gene encoding PTH, is found in a minority of tumors. A high frequency of loss of heterozygosity (LOH) at 1p and 11q in tumors of primary hyperparathyroidism suggests that inactivation of *MEN1* gene and putative tumor suppressor genes at these regions is associated with these diseases.

The molecular pathway of parathyroid oncogenesis is complex and poorly understood. Multiple genetic pathways have been described in primary hyperparathyroidism (Fig. 6), and new insights will help to better understand these diseases.

Primary Parathyroid Hyperplasia Multiglandular involvement of more than two parathyroid glands without a known cause is known as primary parathyroid hyperplasia. There are two distinct types of hyperplasia: chief cell type and clear cell hyperplasia.

Chief cell hyperplasia, caused by an undefined stimulus, is characterized by diffusely or nodular enlarged glands, with decreased fat. Clear cell hyperplasia causes pronounced symptoms of hyperparathyroidism, with markedly enlarged glands.

The findings associated with this disorder are more commonly 11q13 loss, somatic mutation of the *MEN1* gene, and reduced calcium-sensing receptor, with loss of 3q. There is overexpression of cyclin D1 and retinoblastoma protein [77,79–82].

Vitamin D plays a crucial role in the regulation of the parathyroid glands. Vitamin D receptor (VDR) *A*, *B*, and *T* alleles are overrepresented in primary hyperparathyroidism, particularly in postmenopausal female patients [43,83].

Parathyroid Adenoma Parathyroid adenomas are common. They account for approx 85% of cases of primary hyperparathyroidism. Sporadic nonfamilial parathyroid adenomas are the most frequent cause of primary hyperparathyroidism, and important progress has been made in defining the molecular basis of these benign clonal tumors. In this disorder, parathyroid cells divide infrequently, although their ability to proliferate on stimulus is retained.

Clonal endocrine tumor cells divide far more frequently than the polyclonal cell relatives. Parathyroid adenomas are monoclonal lesions. A disproportionate accumulation of one or several clones is a feature of these neoplasms. There are several tests for clonality, including X chromosome inactivation, LOH at a locus, and comparative genome hybridization.

Arnold et al. demonstrated by DNA polymorphism approaches that parathyroid neoplasms are monoclonal. Subsequent studies confirmed these findings. Monoclonality was also demonstrated in parathyroid tumors of MEN 1, some lesions classified as sporadic primary parathyroid hyperplasia. Most pathologi-

Table 1
Differential Diagnosis of Primary Hyperparathyroidism

	Inheritance	*Number of abnormal glands*	*Size*	*Clonality*	*Surgery*	*Pathophysiology*
Sporadic adenoma	Not inherited	One	Markedly enlarged (20×)	Monoclonal or oligoclonal	Curative	Acquired mutations with development of a neoplastic clone
MEN 1	Autosomal dominant	Multiple	Enlarged (5–10×)	Monoclonal or oligoclonal	Curative	Inherited and acquired inactivation of *MEN1* gene with development of neoplastic clones
Familial hypocalciuric hypercalcemia	Autosomal dominant	Multiple	Normal to mild enlargement	Polyclonal	Not indicated	Decreased sensing calcemia levels by inactivation of *CaR* gene
Neonatal severe hyperparathyroidism	Autosomal recessive	Multiple	Very enlarged	Polyclonal	Total parathyroidectomy	Decreased sensing calcemia levels by inactivation of both alleles of the *CaR* gene
MEN 2A	Autosomal dominant	2–3	Enlarged	Not well defined monoclonal	Curative	Inherited and acquired mutations of *RET* gene
Hyperparathyroidism-jaw tumor	Autosomal dominant	Multiple with cysts	Asynchronously enlarged	Monoclonal	Curative	Loss of wild-type of *HRPT2* gene
Familial isolated hyperparathyroidism	Autosomal dominant	Single or multiple	Higher incidence of carcinoma	Monoclonal or oligoclonal	Curative	*HRPT1* gene

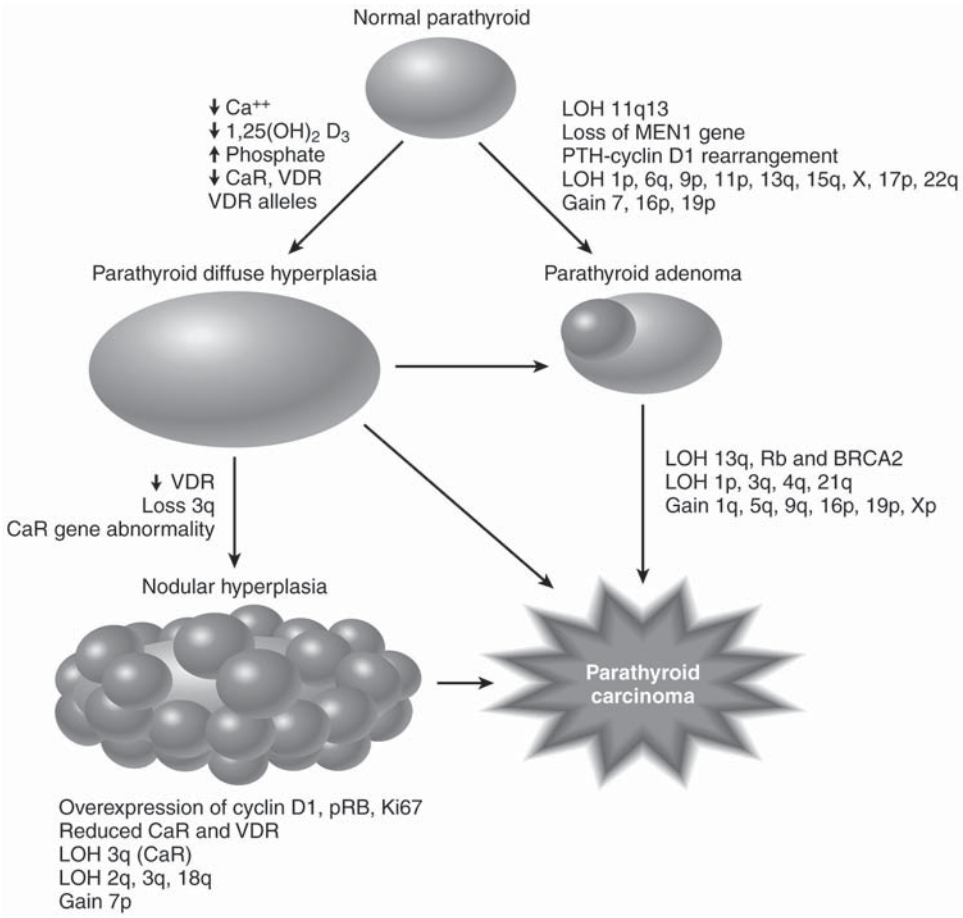

Figure 6 Proposed model of development of the diverse parathyroid disease. Proliferation-promoting factors lead the normal parathyroid to proliferate, which can lead to disease progression. The polyclonal parathyroid hyperplasia can induce a monoclonal proliferation by acquiring somatic mutations, including some losses of genes or gains of chromosomal regions.

cal parathyroid glands were excised at surgery for secondary hyperparathyroidism as a result of renal and parathyroid carcinomas [26,57,82,84–89]. These findings provided the basis for more detailed studies on somatic genetic events causing parathyroid neoplasia. Two parathyroid adenomas bearing clonal restriction fragment abnormalities have been shown to involve

the *PTH* locus. In one of these tumors, the DNA rearrangement occurred at the *PTH* locus [15].

Some parathyroid adenomas were determined to have a reciprocal translocation, in which the PTH gene promoter drives a translocated sequence encoding *cyclin D1*, at 11p15 and 11q13, which indicates a *PTH–cyclin D1* gene rearrangement, and overexppression of *cyclin D1* (Fig. 3).

Cyclin D1 is important for the entry of proliferating cells into the G_1 phase of the cell cycle. D-cyclins regulate the G_1 phase of the cell cycle by inducing the phosphorylation of the retinoblastoma (Rb) tumor suppressor protein, which leads to inactivation of Rb and promotes cellular proliferation. The deregulation or overexpression of *cyclin D1* in a parathyroid cell could accelerate progression from the G_1 into the S phase (Fig. 4). This cyclin would cause excessive cell proliferation, which would not indicate induced malignant phenotype.

Transgenic mice were created recently in which *cyclin D1* was specifically expressed in the parathyroid under the control of a 5.1-kb upstream region of *PTH*. The transgenic mice developed hyperparathyroidism with hyperplasia or adenoma formation with increased PTH secretion. Expression of the CaR protein was decreased in the hyperplastic parathyroid glands, as found on patients with hyperparathyroidism. The decrease of CaR was a secondary phenomenon, and was not the cause of the hyperparathyroidism. These transgenic mice with hyperparathyroidism also developed a high bone turnover with cortical reabsorption [52].

NciI polymorphism has been associated with early onset of hereditary nonpolyposis colorectal cancer and is a prognostic indicator of non-small-cell lung cancer and squamous cell carcinomas [76]. However, there is no pathogenic importance in the development of primary hyperparathyroidism [76].

The *MEN1* gene is a tumor suppressor that encodes Menin, playing an important role in the development of *MEN1*-associated tumors. Somatic *MEN1* gene mutations are also detected in sporadic non-*MEN1* endocrine tumors. Loss of heterozygosity at the *MEN* type I locus of chromosome 11q13 is found in about 25–40% of sporadic parathyroid adenomas, and somatic homozygous mutations of the *MEN1* gene are found in 50% [75, 90]. Inactivating mutations in the tumor suppressor gene Menin (*MEN1*) may produce sporadic adenomas. By comparative genomic hybridization from parathyroid tumors from sporadic cases, cases previously given irradiation to the neck and familial cases showed commonly occurring minimal regions of loss on chromosome 11, 15q15–qter, and 1p34–pter, whereas gains preferentially involved 19p13.2–pter and 7pter–qter. Multiple aberrations were found in sporadic tumors with a somatic mutation and/or LOH of the *MEN1* gene. The irradiation-associated tumors also showed frequent losses of 11q (50%), and subsequent analysis of the *MEN1* gene demonstrated mutations in 50% of the cases [75].

MEN1, as well as another possible 11q13-tumor suppressor gene, can contribute to parathyroid tumorigenesis.

Frequent loss of chromosomal arm 1p has been reported in parathyroid adenomas. In a study, sporadic parathyroid adenomas had frequent allelic losses on chromosome 1p, 1p36, and 1p35–p31, identified by deletion mapping, suggesting that 1p is the location of a putative tumor suppressor gene for parathyroid adenoma tumorigenesis. In addition to parathyroid adenomas, other tumors were reported to have allelic losses on chromosome 1p, such as neuroblastoma, oligodendrogliomas, melanoma, colon cancer, breast cancer, and medullary thyroid carcinoma [91–93]. Another more recent study reveals that inactivation of tumor suppressor genes on 1p seems to be an independent phenomenon of inactivation of tumor suppressor genes on 11q, as allelic losses on chromosome 1p were not associated with allelic losses on 11q [94]. Multiple changes in chromosomes are necessary for tumorigenesis of sporadic parathyroid adenomas. The pathways of tumorigenesis concerning inactivation of putative tumor suppressor genes on 1p, however, seem different from the pathways including inactivation of 11q-tumor suppressor [95].

Deletions of 11q23 were also reported in parathyroid adenomas, suggesting the possibility that another tumor suppressor gene may contribute to the pathogenesis.

Frequent chromosomal gains on 16p and 19p were shown in many patients with sporadic parathyroid adenomas by comparative genomic hybridization, suggesting possible oncogenes on chromosomes 16p and 19q [96]. Frequent losses of heterozygosity were reported on chromosomes 1p, 6q, 11p, 11q, and 15q reported by these authors. Other chromosomal losses are in 9p, 13q, and X. These findings also indicate the presence of one or more tumor suppressor genes on these chromosomal arms (Table 2).

Important progress has been made in defining the molecular basis of these benign, clonal tumors. Multiple chromosomal damage is likely to be necessary for tumorigenesis in sporadic parathyroid adenomas (Table 3).

Accumulated data also show that *cyclin D1* is involved in the development of several different tumor types besides those of the parathyroid. The tumor suppressor *Rb* gene has been linked to the pathogenesis of parathyroid carcinoma. The *MEN1* gene product Menin has been identified, and mutations contribute to sporadic tumors. Mutations in the *RET* gene (*MEN2*) contribute rarely to development of sporadic parathyroid tumors. Mutations in the calcium sensing receptor (*CaR*) gene play a role in familial disease, they do not appear to be involved in sporadic parathyroid tumorigenesis.

Parathyroid Carcinoma Parathyroid carcinoma is an uncommon cause of hyperparathyroidism. It accounts for 1–5.2% of patients with primary hyperparathyroidism [97]. The etiology of parathyroid carcinoma is unclear, although there is a relationship with history of neck irradiation and previous adenoma or hyperplastic parathyroid glands.

Patients with end-stage renal disease can develop parathyroid carcinoma. A recent review of parathyroid carcinoma in 12 end-stage renal disease patients, who between 1982 and 1996 were receiving maintenance hemodialysis [98], demonstrated hyperplasia of other parathyroid glands and one had a history of prior neck irradiation [99].

Carcinoma has been reported in association with familial hyperparathyroidism particularly in the autosomal dominant form with isolated hyperparathyroidism that is not part of the MEN1 syndrome.

Chromosomal abnormalities commonly observed in other solid tumors were identified in a family as translocation of chromosomes 3 and 4, trisomy 7, and a pericentromeric inversion

Table 2
Molecular Differential Diagnosis of Hyperparathyroidism

Cause	*Findings*
Primary:	
Adenoma, sporadic	LOH 11q13, *MEN1* gene, 20–30%
	PTH–cyclin D1 rearrangement, <5%
	LOH 11q13 in 33%, and half of those, mutations
	LOH 1p, 6q, 9p, 11q, 11p, 13q, 15q, 17p, 22q, X
	Gain 7, 16p, and 19p
Carcinoma	LOH 13q, with tumor-specific loss *Rb*, abnormal expression *Rb*
	LOH 13q with loss *BRCA2*
	LOH of P53, abnormal expression of the protein p53
	Gains 1q, 5q, 9q, 16p, 19p, Xp
	LOH of 1p, 3q, 4q, 13q, 21q
Primary hyperplasia	LOH 11q13, somatic mutation *MEN1*
	Reduced *CaR* with loss 3q
	Reduced vitamin D receptors
	Overexpression *pRb*, *cyclinD1*, *Ki67*
MEN 1	A.D., germline mutation 11q13, *MEN1*
MEN 2A	A.D., germline mutation 10q21, *RET*
Familial isolated hyperparathyroidism	A.D., 1q21–q23 (*HPT-JT* or *HRPT2* locus)
	MEN1, 11q13
Hyperparathyroidism-jaw tumor	A.D., 1q21–q23 (*HRPT2* locus)
	LOH 1q, 1p, 11q13
Familial hypocalciuric hypercalcemia	A.D., 3q13.3–q21 (*CaR*)
	19p13.3 (?gene)
	19q13 (?gene)
Autosomal dominant mild hyperparathyroidism	A.D., 3q13.3–q21 (*CaR*)
Neonatal severe hyperparathyroidism	A.R., 3q13.3–q21 (*CaR*)
Familial hypercalcemia	A.D., 3q13.3–q21(*CaR*)
Secondary and tertiary	
Diffuse hyperplasia and/or nodular hyperplasia and/or superimposed adenoma or carcinoma	Greater expression of *cyclin D1, pRb, Ki67* in nodular hyperplasia than in diffuse hyperplasia
Chronic renal failure	*CaR* reduced in nodular hyperplasia
Malabsorption	*CaR* gene abnormality (LOH 3q in 10%)
Vitamin D deficiency	Reduced vitamin D receptors
Renal tubular acidosis	

in chromosome 9 [100]. There was no evidence of *ras* gene mutations, *PTH* gene arrangement, or allelic loss from chromosome 11q13 in one of their patients studied.

In addition, a greatly increased risk of parathyroid carcinoma is associated with the hereditary hyperparathyroidism–jaw tumor syndrome, related to 1q21–q31 [101–103].

Familial hyperparathyroidism and parathyroid carcinoma are rare, and further studies should clarify their relationship. A case of parathyroid carcinoma in an 8-yr-old girl whose mother had previously undergone parathyroidectomy for primary hyperparathyroidism suggests that it may have a familial basis [104].

Cyclin D1 is an oncogene involved in parathyroid adenomas. Overexpression of cyclin D1 protein is frequent in parathyroid carcinomas, having been identified in 91% of such tumors in one study [77] and in two of three in another [78]. There is a strong suggestion that *cyclin D1* overexpression is a feature of parathyroid carcinoma.

Six parathyroid carcinomas and their metastases in comparison with parathyroid adenomas and hyperplasia were analyzed for *cyclin D1*, both by immunohistochemistry and by FISH [105]. All carcinomas demonstrated overexpression of *cyclin D1* by immunohistochemistry (Figs. 7 and 8). Using FISH in paraffin sections, confirmation of polysomies of chromosome 11 and *cyclin D1* in the neoplastic cells (Fig. 8). *Cyclin D1* might prove to be a therapeutic target for this disease.

As with *cyclin D1*, the tumor suppressor gene retinoblastoma (*Rb*) is important in cell cycle control. Immunohistochemical staining of Rb protein can help in distinguishing benign from malignant parathyroid tumors. Rb protein is usually absent in parathyroid carcinomas and is present in parathyroid adenomas. Some authors did not find immunostaining of Rb protein to be useful in distinguishing between these lesions [106]. Strong evidence exists for the presence of a gene on chromosome 13q whose acquired inactivation contributes to the development of parathyroid carcinoma (Table 2). Parathyroid carcinomas were investigated for evidence of loss of a region on chromosome 13 containing Rb and for altered expression of Rb protein [68]. All parathyroid malignancies lacked a *Rb* allele, and most had complete absence of nuclear staining for the Rb protein. Allelic losses of *Rb* or *D13S71* at 13q14 in a parathyroid carcinoma have also been reported [69]. Loss of 13q is found frequently in parathyroid carcinomas [107].

An allelic deletion of the 13q12–14 region involves also the hereditary breast cancer susceptibility gene (*BRCA2*). It was

Table 3
Possible Genes Involved in Parathyroid Diseases

Gene/location	*Protein*	*Function/defect*	*Lesion*	*Evidence*
MEN1: 11q13	Menin	Tumor suppressor	Hyperplasia Adenoma	LOH in adenomas *MEN* mutations
Cyclin D1: 11q13	Cyclin D1	Cell cycle regulation	Adenoma Carcinoma Secondary HPT	Rearrangement 11q13 and 11p15 Amplification of cyclin LOH in secondary HPT
CaR: 3q13.3–q21	Calcium receptor	Calcium-sensing receptor	Hyperplasia Adenoma Familial Hypocalciuric Hypercalcemia Neonatal severe HPT	LOH in adenomas and secondary HPT One mutation: familial hypocalciuric Hypercalcemia Two inactivating mutations: neonatal severe HPT
Rb: 13q14	Rb	Tumor suppressor Cell cycle regulation	Carcinoma	LOH *Rb* in carcinomas Immunoreactive *Rb* detectable
RET: 10q11.2	RET tyrosine kinase	Oncogene	Hyperplasia Adenoma	Somatic mutation *RET*
HRPT2 gene: 1q21–q32	HRPT2	Tumor suppressor	Familial isolated hyperparathyroidism-jaw tumor	LOH in adenomas LOH in familial isolated HPT and HPT-JT
? 11q23	Unknown	Tumor suppressor	Adenoma	LOH 11q23 is frequent in adenomas
p53: 17p13.1	P53	Tumor suppressor	Carcinoma Adenoma	LOH p53 Point mutation p53
? 1p35–36	Unknown	Tumor suppressor	Adenoma	LOH 1p35–36 frequent
Vitamin D receptor			Adenoma	Polymorphisms
BRCA2 13q		Tumor suppressor	Familial isolated hyperparathyroidism	LOH 13q in familial isolated HPT
1q, 5q, 9q, 16p, 19p	Unknown	Oncogene	Carcinoma	Gains in carcinoma
1p, 3q, 4q, 13q, 21q 6q, 9p, 15q	Unknown Unknown	Tumor suppressor	Carcinoma	Losses in carcinoma

found in 3 of 19 parathyroid adenomas that had aggressive features and in 1 parathyroid carcinoma. In parathyroid carcinoma, it remains to be determined whether *Rb*, *BRCA2*, or a different gene will be the primary causative tumor suppressor [66].

These data strongly support the presence of a tumor suppressor gene on the long arm of chromosome 13, which is critical for the development of parathyroid carcinoma.

The *p53* tumor suppressor gene, located at 17p13.1, is another important cell cycle regulator and a candidate for involvement in parathyroid carcinomas. However, its frequency of p53 allelic loss and abnormal p53 protein expression (Fig. 7) is low [64,67]. It appears that *p53* does not play a major role to the pathogenesis of parathyroid carcinoma.

Potentially important oncogenes or tumor suppressor genes were reported in two series of parathyroid carcinomas [107, 108]. Several recurrent abnormalities that seem to be preferentially or exclusively appear in carcinomas compared with adenomas were found on some studies [66–69,100,107,109,110]. Tumor-specific gains suggested that oncogenes in locations including 1q, 5q, 9q, 16p, 19p, and Xq, or losses of chromosomal material, suggested that tumor suppressor genes in locations including 1p, 3q, 4q, 13q, and 21q might be involved in the pathogenesis of parathyroid carcinoma (Table 2). The regions commonly lost in adenomas, such as 11q13, were rarely lost in carcinomas [63,65,97,101,104,105,107,108,111].

These findings support the hypothesis that parathyroid carcinomas arise *de novo* rather than from preexisting adenomas. The difficult differential diagnosis between parathyroid hyperplasia and neoplasia will benefit from clarification of the molecular pathogenesis of these lesions.

FAMILIAL HYPERPARATHYROIDISM Familial hyperparathyroidism is usually part of multiple endocrine lesions. The usual histology in the hyperparathyroidism is primary chief cell hyperplasia. It also occurs in families without evidence of other endocrine disease. The diagnosis of chief cell hyperplasia should be followed by the study of other organ involvement in the patient and in the family.

MEN is characterized by involvement of two or more endocrine glands by neoplasia. These syndromes are rare. There are two major forms, referred to as MEN 1 and MEN 2. Parathyroid,

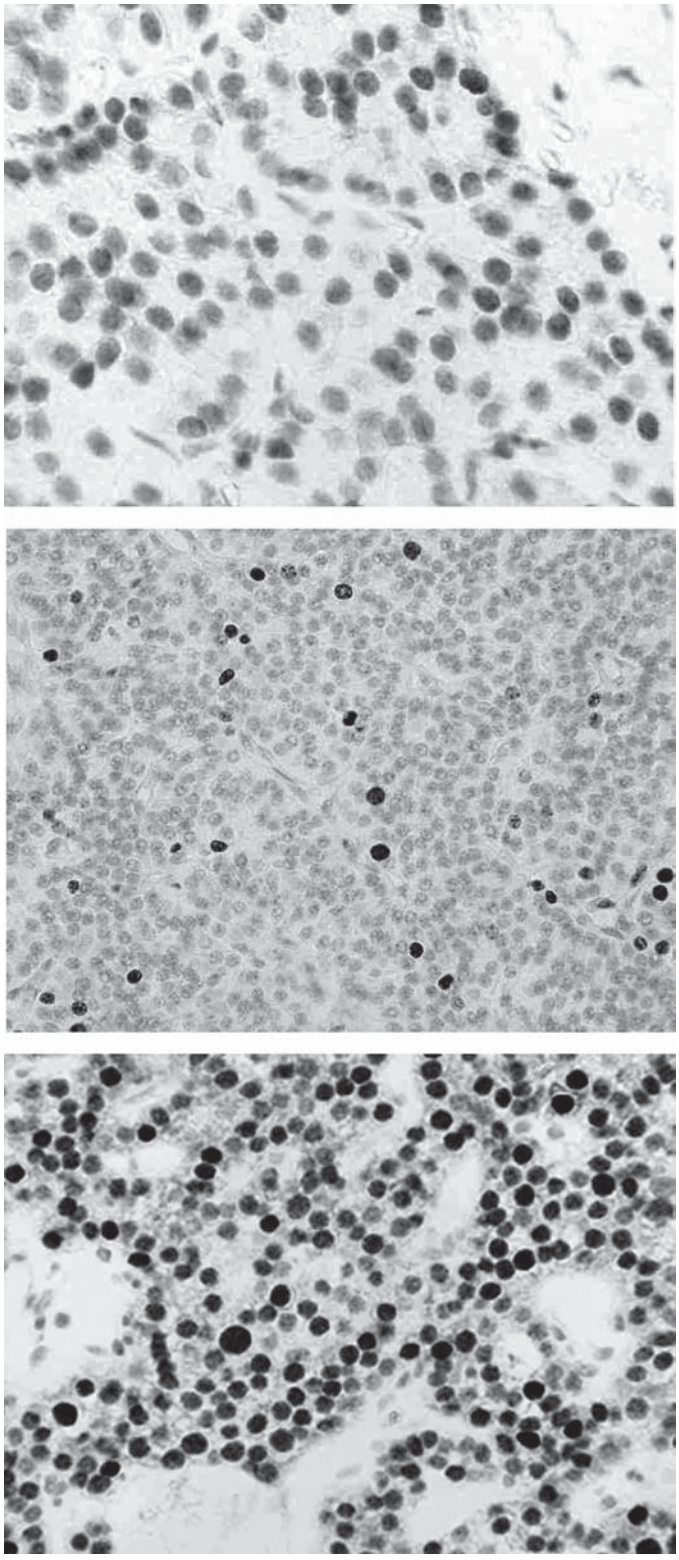

Figure 7 Immunohistochemistry for *p53*, *Ki67*, and *Cyclin D1* in parathyroid carcinoma. *p53* was observed in parathyroid carcinomas (**top**). There is a higher proliferative activity index, demonstrated by *Ki67* immunostain, in parathyroid carcinomas, compared with parathyroid adenomas and hyperplasia (**center**). An usual finding in parathyroid carcinoma is overexpression of *cyclin D1* (**bottom**). (Color illustration appears in insert following p. 148.)

pancreatic islet, and anterior pituitary tumors characterize MEN 1. Nearly all patients will develop parathyroid neoplasia in this syndrome by the age of 50. MEN 2 is characterized by medullary thyroid carcinoma (MTC) in association with pheochromocytoma. There are three clinical variants of MEN 2, referred to as MEN 2A, MEN 2B, and MTC-only. All these forms of MEN may be inherited as autosomal dominant syndromes.

Many others inherited familial disorders have been reported. The knowledge of tumorigenesis in inherited parathyroid disease led to the description of hyperparathyroidism-jaw tumor syndrome, familial hypocalciuric hypercalcemia, neonatal severe hyperparathyroidism, familial isolated hyperparathyroidism, and more recently, the autosomal dominant mild hyperparathyroidism (Tables 1, 2, and 4).

MEN 1 MEN 1, first described in 1954 [4], is an autosomal dominant disease caused by mutation of the *MEN1* gene. This gene has been implicated in multiple nonendocrine and endocrine neoplasia. The *MEN1* gene is located in chromosome 11q13 (Fig. 4). It is a tumor suppressor gene. Patients affected by familial MEN 1 inherit one inactive *MEN1* allele. With inactivation of the remainder allele in specific tissue, tumorigenesis occurs. This can be recognized in tumor DNA as LOH at the *MEN1* locus at 11q13. Tumor development in *MEN1* follows Knudson's two-hit hypotheses. The germline mutation is present in every cell in the patient with MEN 1 syndrome, while the deletion of a wild-type allele is present only in MEN tumors.

The *MEN1* gene encodes a 610-amino-acid protein called Menin. Menin is located mostly in the nucleus. Its function is supposed to be suppression of the activity of a protein that inhibits growth (Fig. 2) [29]. The interaction between JunD and Menin has yet to be elucidated [112].

The prevalence of MEN 1 varies from 0.01 to 2.5 per thousand. The clinical and pathological features of MEN 1 tumors are related to hormone hypersecretion and malignancy, and are similar to those of a sporadic neoplasia of the same organ. This syndrome is defined by the presence of multiple endocrine organ neoplasia, occurrence at an earlier age than similar sporadic cases, and malignant potential.

MEN 1 predisposes to tumor development in a variety of tissues, and is expressed mainly as parathyroid, enteropancreatic gastrin neuroendocrine, and pituitary tumors. Primary hyperparathyroidism is the usual manifestation of the disorder. Primary hyperparathyroidism is present in 80–100% of patients with the syndrome. Gastrin secreting tumors are the major cause of morbidity and mortality. Gastrinomas are usually multiple and malignant. Insulinomas occur in up to 35% of patients. Prolactinomas are the most common pituitary tumor, and are present in 50% of MEN 1 patients [25,53,113–115].

MEN-related multiple facial angiofibromas, collagenomas, or lipomas had allelic loss at the *MEN* locus. These findings indicate that these neoplasms are clonal and caused by inactivation of both *MEN* alleles [53].

Adrenal cortical tumors are common in MEN 1. Smooth muscle tumors, as well as thyroid follicular neoplasms that are MEN 1 related are not confirmed [116]. Pheochromocytomas are rare in MEN 1.

As in the familial *MEN1*-associated tumors, sporadic tumors may develop as a result of *MEN* germline mutations associated

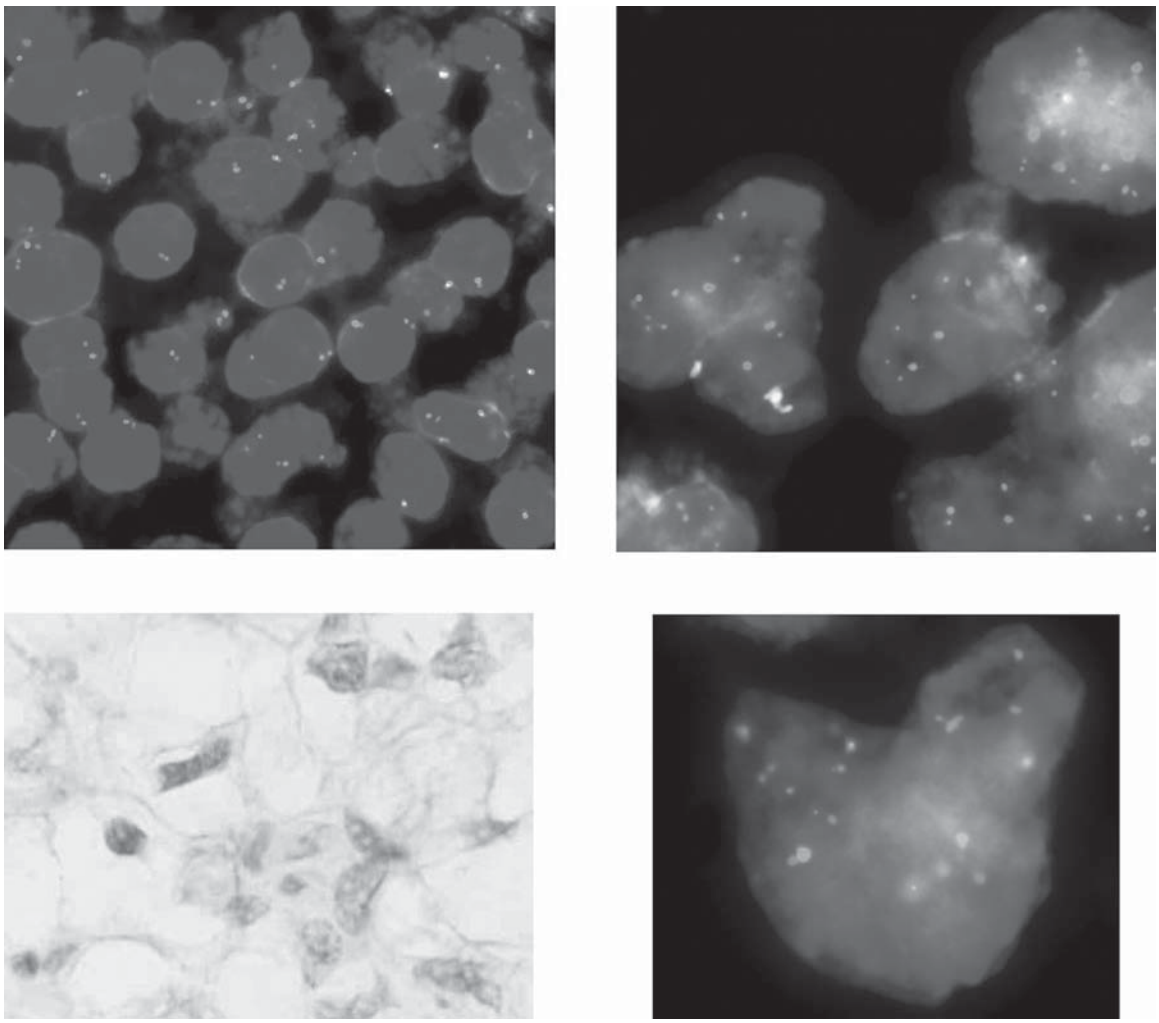

Figure 8 FISH for chromosome 11 and *cyclin D1* in parathyroid adenoma and carcinoma. Immunohistochemistry for *cyclin D1* in metastatic carcinoma. Paraffin-embedded sections of parathyroid neoplasm demonstrating up to two copies of chromosome 11 and *cyclin D1* in adenomas (up to two red and green signals), **top left**. Primary and metastatic carcinoma showing polysomies of chromosome 11 and *cyclin D1* (multiple red and green signals) as well as expression of *cyclin D1* by immunohistochemistry. (Color illustration appears in insert following p. 148.)

with somatic LOH loss at 11q13. Somatic mutations in sporadic parathyroid adenomas have been reported as high as 22% [62, 73,117,118]. Other sporadic tumors associated with these mutations are gastrinomas, insulinomas, lipomas, angiofibromas, vasoactive intestinal peptide secreting tumors, and lung and gastric carcinoids.

MEN 2A MEN 2A, or Sipple syndrome, is an autosomal dominant disease. This disorder is characterized by primary parathyroid hyperplasia and hyperparathyroidism, pheochromocytoma, and C-cell hyperplasia/medullary thyroid carcinoma. The hyperparathyroidism is caused by multiglandular parathyroid hyperplasia or adenoma, and is present in about 20% of cases. Medullary thyroid carcinoma, however, is seen in almost all patients with inherited *MEN2A* gene [119].

MEN 2A results from an activating mutation of the *RET* proto-oncogene, localized at chromosome 10q21. The protein coded by the gene *RET* is in the plasma membrane, *RET*-encoded tyrosine kinase with extracellular domains, and a tyrosine kinase intracellular domain involved in cell growth and differentiation. Some versions of the rearrangement of the protooncogene *RET*, called *RET/PTC*, are specific markers of papillary thyroid carcinoma. This rearrangement leads to the formation of chimeric oncogenes. Similar rearrangements with have not being identified in parathyroid lesions.

The germline mutation of the *RET* in MEN 2A result in a gain of function, different from other inherited diseases. Those diseases are result of loss of function mutations that inactivate tumor suppressor proteins, as seen in Hirschsprung's disease.

There are specific mutations of c-*RET* for each of the three MEN 2 variants. Mutational analysis of condons 609, 611, 618, 620 (codon 10), and 634 is often sufficient to identify the *RET* mutation in patients of the MEN 2A family. The majority of mutations in MEN 2A involve one of these five cysteine residues in the cysteine-rich region of the RET protein extracellular domain encoded in *RET* exons 10 or 11. A mutation at condon 634 (codon 11), Cys to Arg, is found preferentially in families of MEN 2 syndrome with hyperparathyroidism [119], and it is the most common genetic finding in this disease. The pene-

Table 4
Familial Syndromes with Hyperparathyroidism

Syndrome	*Clinical features*	*Chromosome location*	*Gene/protein*	*Function/defect*
MEN 1	Multiglandular parathyroid hyperplasia, pancreas, pituitary tumors	11q13	*MEN1*/ Menin	Tumor suppressor gene/ Mutations in 90% of MEN families Familial:Inherited mutation plus inactivation of a second copy. Sporadic: Two sequential inactivating somatic mutations.
MEN 2A	C-cell hyperplasia/neoplasia, pheochromocytomas, parathyroid hyperplasia	10q11.2	*RET*	Protooncogene (Tyrosine Kinase mutation)
MEN 2B	C-cell hyperplasia/neoplasia, pheochromocytomas, GI ganglioneuromatosis, rare parathyroid hyperplasia	10q11.2	*RET*	Protooncogene (Tyrosine Kinase mutation)
Hereditary hyperparathyroidism-jaw tumor syndrome	Solitary parathyroid adenoma/ carcinoma, fibroosseous jaw lesions, Wilms polycystic kidney disease, renal hamartomas	1q21–q32	*HRPT2* locus: gene to be cloned	Tumor suppressor gene
Familial isolated hyperparathyroidism	Solitary or multiple adenomas/ carcinomas, hypercalcemia	1q21–q32 11q13	*HPT-JT* *MEN1*	Tumor suppressor gene
Familial hypocalciuric hypercalcemia	Hypercalcemia with normal parathyroid histology, mild enlargement of parathyroid, inappropriate serum PTH	3q13–21 19p13.3 19q13	*CaR*	Tumor suppressor gene/ heterozygous inactivating mutation
Neonatal severe hyperparathyroidism	Marked parathyroid hyperplasia, symmetric, diffuse	3q13–21	*CaR*	Tumor suppressor gene/ homozygous mutations
Autosomal dominant mild hyperparathyroidism	Hyperplasia/adenoma, hypercalcemia, hypercalciuria	3q13–21	*CaR*	Tumor suppressor gene/atypical inactivating mutation in the intracellular portion
Familial hypercalcemia hypercalciuria	Hyperplasia, adenoma	3q13–21	*CaR*	Tumor suppressor gene

trance of hyperparathyroidism in the patients with mutation at condon 634 is approx 20%. *RET* mutations have not been identified in sporadic parathyroid adenomas. Mutations in exon 13 of the *RET* (codons 790 and 791) occur less often [63]. Mutations in condon 768 of exon 13 is seen only in MEN 2 B. The small number of sites at the *RET* in which this limited number of known mutations are seen is an advantage for the molecular studies of this disease.

Different mutations in the *RET* gene leads to unrelated disorders, demonstrating the value of the site of the mutations and its effect for the development of unrelated diseases.

Hyperparathyroidism is not seen in the other types of MEN 2—the MEN 2B, FMTC, and sporadic medullary carcinoma.

Hereditary Hyperparathyroidism and Jaw Tumor (HPT-JT)

Jackson first reported a family with hereditary hyperparathyroidism associated with jaw tumors [120]. The maxillary and mandibular tumors could be differentiated from the brown tumors of hyperparathyroidism (ossifying fibroma of the jaw). Parathyroid enlargement was mostly adenoma with occurrence of cysts. Other diseases have been described in this syndrome, such as renal hamartomas, Wilms tumor, polycystic kidney disease, and cysts. Evidence that parathyroid carcinoma and Wilms' tumor are part of the HPT-JT syndrome came from a report in 1994 [102].

The hyperparathyroidism and jaw tumor syndrome is an autosomal dominant familial disorder, linked to the chromosomal region of 1q32–q21 (HRPT2 locus) [121–123]. Two families with HPT-JT syndrome in which adult renal hamartomas or cystic kidney disease were associated features. Seven renal hamartomas showed LOH in the 1q21–q32 region, suggesting the occurrence of inactivation of a tumor suppressor gene in this region (HRPT2 locus) [124].

There is no clear association of this syndrome with chromosome 11p13 (MEN 1), or chromosome 10q11.2 (MEN 2).

In cystic sporadic parathyroid adenomas of HPT-JT, LOH was found on 1q, on 1p, and on 11q13 [125]. Some authors found no association of changes in 1q, 11q, and X in the cases of hyperparathyroidism-jaw tumor in one family [126].

Thirteen affected members of a family presented with either parathyroid adenoma or carcinoma. In five affected individuals, cystic kidney disease was found, in addition to pancreatic adenocarcinoma, renal cortical adenoma, papillary renal cell carcinoma, testicular mixed germ cell tumor, and Hurthle cell adenoma of the thyroid, excluding the involvement of the *MEN1* gene [60].

There is a suggestion that cystic parathyroid tumors might represent a different subgroup among parathyroid neoplasia.

Familial Isolated Hyperparathyroidism (FIH) Familial hyperparathyroidism is usually part of endocrine adenomatosis. FIH, without association of other tumors, has been described as a separate entity. These patients present with profound hypercalcemia more frequently as compared with MEN 1. There is a tendency toward malignant transformation in the glands. Whether FIH is a variant of MEN 1 or hyperparathyroidism–jaw tumor syndrome is yet to be established [103,117,127].

In parathyroid tumors from FIH families, repeated allelic losses of the *HRPT2* region in 1q21–32, but these losses are infrequent in sporadic hyperparathyroidism [51,121].

Some authors found no *MEN1* germline mutation [118]. However, another cause of FIH is mutation in the *MEN1* gene [117].

Analysis of tumor DNA from patients from a single family with FIH, from the parathyroid tumors, showed limited loss of heterozygosity on 9p22–p21 and 13q12.3–q32 [65]. There is a suggestion of a possible contribution of tumor suppressor genes, as retinoblastoma gene and the hereditary breast cancer susceptibility gene (*BRCA2*) on 13q associated with the parathyroid tumors in this family.

Screening for other tumors associated with MEN 1 and HPT-JT should be performed for the diagnosis of FIH. More genetic studies of FIH families are needed to clarify better the genetic basis of this disease.

Familial Hypocalciuric Hypercalcemia (FHH) Hypercalcemia and hypercalciuria and familial benign hypocalciuric hypercalcemia (FHH) characterize familial hyperparathyroidism. This is the most common cause of hereditary hypercalcemia. FHH is inherited as an autosomal dominant trait with mild to moderate hypercalcemia, accompanied by few symptoms. The condition does not require treatment, and responds poorly to parathyroidectomy (Table 1) [128]. Familial hypocalciuric hypercalcemia prevails in about half of cases of hypercalcemia during the first two decades of life.

Loss-of-function mutations in the *CaR* gene are responsible for this disease [19,129], as well as for neonatal severe hyperparathyroidism. Gain-of-function mutations result in autosomal dominant hypocalcemia, discussed later [129–133].

A form of autosomal dominant hypoparathyroidism linked to a region of 3q13 suggested that it might be caused by an inactivating mutation in the *CaR*, with suppression of PTH secretion and lowering the set point for serum calcium levels [134].

FHH should be distinguished from other hypercalcemic disorders such as primary hyperparathyroidism, in which the elevated serum and urinary calcium levels are normalized by successful parathyroid surgery.

Neonatal Severe Hyperparathyroidism Neonatal severe hyperparathyroidism is a rare disorder characterized by extreme hypercalcemia due to diffuse chief cell hyperplasia, with bony changes with progressive demineralization and pathologic fractures. Total parathyroidectomy in the neonatal period is necessary for survival of the patient.

Neonatal hyperparathyroidism is an aggressive expression of hyperparathyroidism and it is caused by loss-of-function mutations in the CaR [23,132]. There are demonstrations that heterozygous mutations in the can also cause the disease [19, 135]. The authors conclude that dosage of the gene defect accounts for the different phenotypes; a single defective allele causes familial hypocalciuric hypercalcemia, while two defective alleles causes neonatal severe hyperparathyroidism.

Neonatal hyperparathyroidism and hypocalciuric hypercalcemia are apparently different manifestations of one mutation of the *CaR* gene.

Other Conditions The inherited parathyroid tumor susceptibility disorders in progress studies are helping and will keep helping our understanding of tumorigenesis. Autosomal dominant inheritance is found in several inherited disorders such as MEN 1, MEN 2A, hereditary hyperparathyroidism-jaw tumor syndrome, and familial isolated hyperparathyroidism (Table 1).

New phenotypes, different from the now known disorders, have been reported, as autosomal dominant mild hyperparathyroidism [136]. This is cause by a mutation in the cytoplasmic tail of the calcium receptor. Autosomal dominant mild hyperparathyroidism is rare, described in a large family suffering from hypercalcemia, hypercalciuria, and normal serum parathyroid hormone. Some of the cases reported presented with parathyroid adenoma or hyperplasia, with improvement of the hypercalcemia after surgery. In these cases the inactivating mutations in the intracellular part of the CaR is associated with the development of parathyroid neoplasia. Familial hypercalcemia and hypercalciuria has been described, associated wit parathyroid hyperplasia or adenoma. This rare disorder is associated with mutations of the *CaR* (Tables 1, 2, and 4).

SECONDARY AND TERTIARY HYPERPARATHYROIDISM

Chronic renal failure, hypocalcemia, active vitamin D deficiency, and phosphate retention stimulate the synthesis and secretion of PTH and proliferation of parathyroid cells (Fig. 5). Reduction of VDR and CaR also contributes to secondary hyperparathyroidism [45,71,72,137–139]. However, the mechanisms that regulate the number of parathyroid cells and how these stimuli contribute to parathyroid cell hyperplasia are not completely understood. The stimuli that can drive the parathyroid cell to leave the G_0 and enter the cell cycle are the continuous low serum calcium or high serum phosphate levels. These are considered the major factors for the parathyroid cell proliferation. Vitamin D therapy decreases PTH transcription and parathyroid cell proliferation through its effect on circulating calcium levels. On the other hand, vitamin D deficiency with secondary chronic hypocalcemia can cause parathyroid cells to proliferate.

A complex homeostatic system exists in humans for the maintenance of a tightly regulated serum calcium concentration. The fluctuations in extracellular calcium concentration are detected by the parathyroid cells, which respond with rapid changes in the secretion of PTH. It has been demonstrated that

this capacity is mostly mediated via the CaR expressed in the parathyroid cells, C cells of the thyroid, and by the renal cells. Thus, the CaR is activated by increase in serum calcium and activates second messengers that lead to a decrease in PTH secretion. With hypocalcemia the CaR is relaxed and PTH secretion is not restrained. High serum phosphate levels also increase PTH secretion independently of changes in serum calcium or serum 1,25(OH)-vitamin D levels. The effect of phosphate on the parathyroid is mediated by phospholipase A_2. Parathyroid cell proliferation is increased by chronic hyperphosphatemia and dramatically decreased by hypophosphatemia.

Secondary hyperparathyroidism is associated mostly with renal failure, and the hypocalcemia and hyperphosphatemia induce an increase in PTH. The development of autonomous gland on patients with secondary hyperparathyroidism is better referred to as tertiary hyperparathyroidism.

Some parathyroid glands from patients with uremic refractory secondary/tertiary hyperparathyroidism show monoclonal neoplasms [70,85,89]. Evidence exists that each nodule is of monoclonal origin and that the monoclonal origin of each nodule is independent [140].

Overexpression of *cyclin D1* was not seen in secondary parathyroid hyperplasia [70,89], while it was found in primary adenomas. Nodular hyperplasia showed greater expression of *cyclin D1*, *pRb*, and *Ki67* when these cases were compared with diffuse hyperplasia (Fig. 6) A higher prevalence of *p53* expression in secondary hyperparathyroidism suggests that *p53* might be involved in some of these cases [64].

The CaR was seen reduced in nodular hyperplasia as compared with diffuse hyperplasia, in both mRNA and protein concentrations [141].

A large group of uremia-associated parathyroid tumors were studied to determine the genetic abnormalities that underlie this clonal expansion of this disorder. By comparative genomic hybridization (CGH), one or more chromosomal changes were present in 24% of the tumors, different from the value for common sporadic adenomas (72%). By CGH gains on chromosomes 7 and 12 were observed. Losses on chromosome 11 occurred in only one of the 46 uremia-associated tumors; the tumor also contained a somatic mutation of the remaining *MEN1*. A total of 13% of tumors demonstrated recurrent allelic loss on 18q. Demonstrations of recurrent clonal DNA abnormalities suggest the existence and locations of genes important in uremic hyperparathyroidism. These findings indicate different molecular pathogenetic processes exist for clonal outgrowth in severe uremic hyperparathyroidism vs sporadic parathyroid adenomas [85].

Possible oncogenes located on chromosomes 7 and 12 and tumor suppressor genes on chromosome 18q may be involved in secondary hyperparathyroidism.

Different molecular pathogenic processes within the uremic hyperparathyroidism and primary hyperparathyroidism are responsible for the clonal growth of the parathyroid cells. (Table 2; Figs. 5 and 6)

Numerous genetic abnormalities are involved in parathyroid tumorigenesis. Various chromosomal regions, various loci containing oncogenes, tumor suppressor genes, and genes for the calcium receptor are implicated in the development of parathyroid tumors. Advances in molecular genetics have shed important new light on our understanding of the parathyroid lesions, mostly the monoclonal proliferation and other nonclonal abnormalities.

Parathyroid Cysts Parathyroid cysts are rare. A parathyroid cyst may present as a neck mass or be discovered as an incidental finding during neck surgery or imaging, or can present in patients with hyperparathyroidism usually due to cystic degeneration of a parathyroid adenoma. These cysts can be divided in three groups according to the mechanism of cyst formation: as developmental, arising from vestigial remnants of the third and fourth branchial clefts; coalescence of microcysts into macrocysts or degeneration of an adenoma, or rarely carcinoma, into a pseudocyst. No specific molecular changes are found in these cases.

MOLECULAR GENETICS OF HYPOPARATHYROIDISM

HYPOPARATHYROIDISM—INTRODUCTION Hypoparathyroidism refers to an absent or decreased production of PTH conducting to low serum calcium levels. Hypocalcemia and hyperphosphatemia characterize this condition.

Hypoparathyroidism can be congenital or acquired. Neonatal hypoparathyroidism can be associated with disorders of hypoplasia or aplasia of the third and fourth branchial pouches. Acquired hypoparathyroidism usually results from surgical procedures or radiation therapy.

Hypothyroidism can be familial or isolated. Familial hypoparathyroidism is a heterogeneous group of disorders of different inheritance patterns. Isolated hypoparathyroidism can occur alone or in a familial pattern. Different modes of inheritance arise among different families (Table 5).

CONDITIONS ASSOCIATED WITH HYPOPARATHYROIDISM

CaR Mutations The CaR plays an essential role in sustaining ion homeostasis and in patients with mutation of the CaR and with idiopathic hypoparathyroidism there are detectable low levels of PTH, hypocalcemia, and hyperphosphatemia [142–145].

Occasionally, patients with apparently sporadic idiopathic hypoparathyroidism have an activating mutation of the CaR. The hypocalcemia in these cases can range from mild to severe. Distinct activating mutations in the *CaR* gene have been associated with autosomal dominant hypocalcemia and sporadic hypocalcemia [21,142,143,146].

Autosomal dominant hypoparathyroidism associated with short stature and premature osteoarthritis has been reported in a family [147]. However, the sequence alteration in the coding region of the *CaR* gene was seen, but no involvement of this gene could be confirmed in the etiology of this syndrome.

The most common form of genetic hypoparathyroidism is autosomal dominant hypocalcemia. Autosomal dominant hypocalcemia is a familial hypocalcemia with urinary calcium excretion that is inappropriately high normal or elevated in the basal state in relation to the serum calcium concentration, and normal serum PTH concentration.

Table 5
Molecular Differential Diagnosis of Hypoparathyroidism

Cause	*Findings*
Neonatal hypocalcemia, transient or part of syndromes	
DiGeorge syndrome	22q11.2–pter
DiGeorge syndrome 2	10p14–p13
Velocardiofacial syndrome	10p14–p13 22q11.2–pter
Late neonatal hypocalcemia: isolated or part of Kearns–Sayre, Kenny–Caffey syndromes	
Autosomal dominant hypoparathyroidism	Activating mutations of *CaR* gene
Sporadic idiopathic hypoparathyroidism	Activating mutations of *CaR* gene
Familial isolated hypoparathyroidism	Mutations of preproparathyroid hormone gene
X-linked recessive hypoparathyroidism	X-linked
Autosomal recessive hypoparathyroidism	Point mutation of *PTH*
Familial isolated hypoparathyroidism	Mutations in *CaR* and *PTH*
Jansen's chondrodystrophy	Activating mutation *PTHr1*
Blomstrand's chondrodystrophy	Inactivating mutation *PTHr1*
Pseudohypoparathyroidism type 1	$Gs_{\alpha 1}$ protein of the adenylyl cyclase complex (*GNAS1*) mutation
Pseudohypoparathyroidism type 1a: Albright's hereditary osteodystrophy	Inactivating mutation in the stimulatory *GNAS1*
Pseudohypoparathyroidism type 1b	?Mutations in the *GNAS1*-imprinting defect of *GNAS1*
Pseudohypoparathyroidism type 2	Defective cAMP-dependent protein kinase
Autoimmune polyglandular syndrome type I	Autoantigen to *CaR*

The autosomal dominant hypocalcemia is associated with an activating mutation in the CaR; as a result, a low serum calcium concentration is perceived as normal, leading to a downward resetting of the PTH–calcium relationship [21]. Serum PTH concentrations are normal, and, in contrast to other causes of hypocalcemia, urinary calcium excretion is normal or high, presumably due to increased activation of the CaR in the loop of Henle.

The diagnosis of autosomal dominant hypocalcemia should be suspected in hypocalcemic patients with normal serum PTH concentrations and with few if any symptoms of hypocalcemia.

In occasional familial cases with symptomatic hypocalcemia, some have low serum PTH concentrations. In these patients, the usual tests do not differentiate this disorder from other forms of hypoparathyroidism. The diagnosis can be confirmed by mutations in the *CaR* gene. The therapy in symptomatic patients is to maintain a serum calcium concentration just sufficient to improve symptoms.

Acquired defects in the CaR have been reported in patients with hypoparathyroidism or hyperparathyroidism, as an expression of the CaR protein may be reduced in adenomas or in chronic renal failure [147].

Autoimmune Disorders

Autoimmune Polyglandular Syndrome The formerly called idiopathic hypoparathyroidism represents several syndromes, both acquired and congenital. Autoimmune hypoparathyroidism is a common feature of polyglandular autoimmune syndrome type 1 are the most common cause of idiopathic hypoparathyroidism. This is a familial disorder.

Acquired hypoparathyroidism (AH) has been considered to result from an autoimmune process but the self-antigens have not been identified.

Two major types of autoimmune polyglandular syndrome are recognized. Hypoparathyroidism is associated primarily with type 1, which includes adrenal insufficiency, Graves' disease, mucocutaneous candidiasis, chronic active hepatitis, hypophysitis, insulin-dependent-diabetes mellitus, among others.

AH has been considered to result from an autoimmune process. From 25 patients with AH, 17 had type 1 autoimmune polyglandular syndrome and 8 were associated with autoimmune hypoparathyroidism [34]. Five of 25 patients with autoimmune hypoparathyroidism had antibodies against an antigen in human parathyroid gland extracts, the CaR. Fifty-six percent of the sera from patients with AH were positive to the extracellular domain of the CaR, whereas none reacted to the intracellular domain. Sera from patients with various other autoimmune diseases as well as normal controls were negative. These authors identified the CaR as an autoantigen in AH [34].

Parathyroiditis Autoimmune parathyroiditis is usually associated with hypoparathyroidism, but occasionally it is associated with hyperplasia. This rare condition is characterized by infiltration of the parathyroid parenchyma by numerous lymphocytes. The clusters of lymphocytes occasionally form lymphoid follicles, with numerous plasma cells.

Parathyroiditis is also considered to be an autoimmune disorder. Patients with idiopathic hypoparathyroidism have autoantibodies to parathyroid tissue.

Genetic Disorders of the PT and PTHr Within this heterogeneous group of diseases, some genetic causes of hypoparathyroidism, such as abnormalities of the *PTH* gene, have been described [148–150]. PTH-deficient hypoparathyroidism hypocalcemic with hypercalciuria of unknown etiology is also known as idiopathic hypoparathyroidism.

Both autosomal dominant and autosomal recessive forms of familial isolated hypoparathyroidism have been related to mutations in the *PTH* gene.

Isolated familial hypoparathyroidism can be the result of a mutation of the signal peptide-encoding region of the preproparathyroid hormone gene on chromosome 11p.

Two defects of the type 1 PTHr are identified. Both disorders have opposite effects, are caused by mutations of the type 1 parathyroid hormone receptor. Blomstrand's chondrodystrophy is caused by inactivating mutations of the type 1 PTH, and is inherited as an autosomal recessive trait. Jansen's chondrodysplasia is caused by activating a mutation of the parathyroid receptor, and is inherited by an autosomal dominant trait [151].

Disorders of the Stimulatory Guanine Nucleotide Binding Protein The parathyroid hormone receptor type 1 acts on a stimulatory guanine nucleotide binding (Gs) protein, encoded by the *GNAS1* gene. The Gs_{α} subunit mediates cAMP stimulation by parathyroid hormone. Mutations of the *GNAS1* gene, which is located on chromosome 20q13.11 and encodes the α-subunit of the stimulatory GTP-binding protein, have been identified in the two types of pseudohypoparathyroidism described [152].

Children with pseudohypoparathyroidism present with hypocalcemia and hyperphosphatemia, but PTH levels are elevated, indicative of resistance to all the actions of PTH.

Pseudohypoparathyroidism type 1 is characterized by a diminished cAMP response to PTH. Two types have been described (types 1a and 1b) as well as pseudopseudohypoparathyroidism. The gene encoding the stimulatory $Gs_{\alpha 1}$ protein of the adenylyl cyclase complex (*GNAS1*) appears to be involved.

Most of the patients with type 1a form have an inactivating mutation in the *GNAS1* [153]. It is inherited as an autosomal dominant trait. Patients with this form have round facies, short stature, and short metacarpal and metatarsal bones, known as Albright's hereditary osteodystrophy. The hyperphosphatemia induced by the renal defect causes hypocalcemia and, thereby, secondary hyperparathyroidism and osteitis fibrosa.

A disorder known as pseudopseudohypoparathyroidism is characterized by Albright's hereditary osteodystrophy without hypocalcemia due to paternal transmission, with normal maternal allele of the *GNAS1* gene: it is a combination of inactivating mutations of *GNAS1* and Albright's osteodystrophy.

The type 1b is characterized by hypocalcemia without the phenotypic abnormalities of Albright's osteodystrophy. The PTH resistance is confined to the kidney resulting in hypocalcemia, hyperphosphatemia, and secondary hyperparathyroidism. In the pseudohypoparathyroidism type 1b no mutations in the *GNAS1*, *PTH*, or *PTHr* genes were identified. No structural defect of *GNAS1* is confirmed [152,154,155]. It is associated with a defective methylation within *GNAS1*.

Pseudohypoparathyroidism type 2 is characterized by a blunted phosphaturic response to PTH. The pathogenesis is resistance to the intracellular effects of cAMP.

The pseudohypoparathyroid diseases appear to represent a heterogeneous group of disorders with *GNAS1* mutations.

Other Conditions

Acquired Hypoparathyroidism A reduction in the physiologic action of PTH can result from decreased secretion or decreased action of PTH. The most common cause of decreased PTH secretion is postsurgical hypoparathyroidism. It can occur after parathyroid or thyroid surgery or radical neck surgery. It is most common after thyroidectomy for thyroid carcinoma. Postsurgical hypoparathyroidism may be transient, with recovery in days, weeks, or months; permanent; or intermittent.

Other causes of acquired hypoparathyroidism, all very rare, include irradiation and storage or diseases of the parathyroid glands such as hemochromatosis, Wilson's disease, or granulomas. Hypomagnesemia and hypermagnesemia can cause functional hypoparathyroidism.

Congenital Hypoparathyroidism There are a number of other forms of congenital hypoparathyroidism besides the dominant hypoparathyroidism. One form of autosomal dominant hypoparathyroidism is characterized by a mutation in the signal peptide sequence of preproPTH, so that it cannot be processed to PTH normally. The other is associated with renal dysplasia and sensorineural deafness; the molecular defect is not known, and the *PTH* gene is considered normal [156].

Several families with autosomal recessive hypoparathyroidism have been identified, and two families with X-linked recessive hypoparathyroidism have been reported.

Hypoparathyroidism due to parathyroid aplasia or hypoplasia is one component of DiGeorge's syndrome together with thymic aplasia or hypoplasia and cardiac malformations. Most cases are sporadic, but familial cases with autosomal dominant inheritance have been reported. Most patients have microdeletion of part of chromosome 22(22q11.21–q11.23) or a translocation also involving 22q11, t(2;22)(q14;q11) [157,158].

Hypothyroidism, gonadal failure, hypopituitarism, and diabetes mellitus, in association with myopathic abnormalities, characterize Kearns–Sayre syndrome. The disease is also characterized by abnormal inclusions in the mitochondria and ragged red fibers, with antibodies to skeletal muscle.

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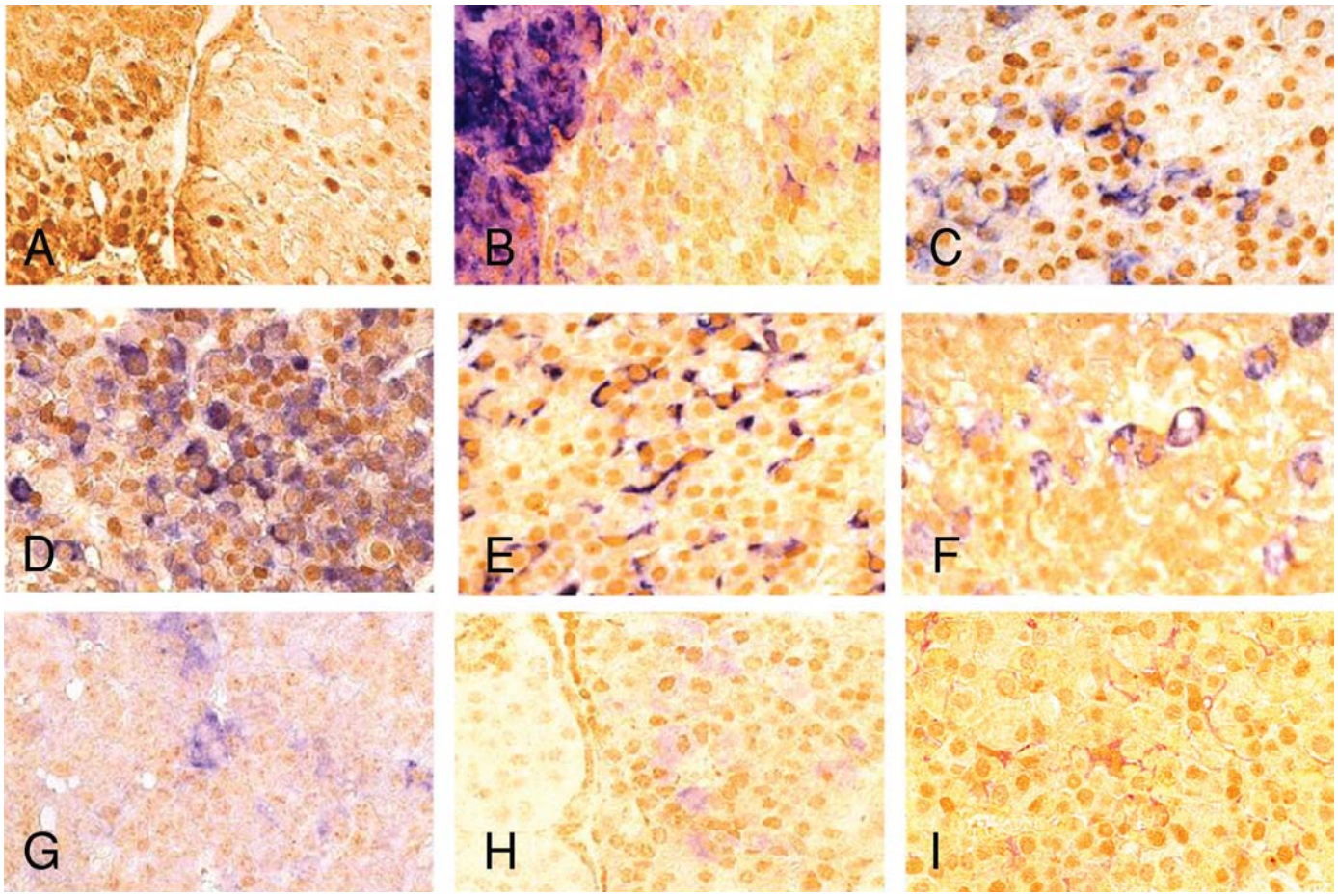
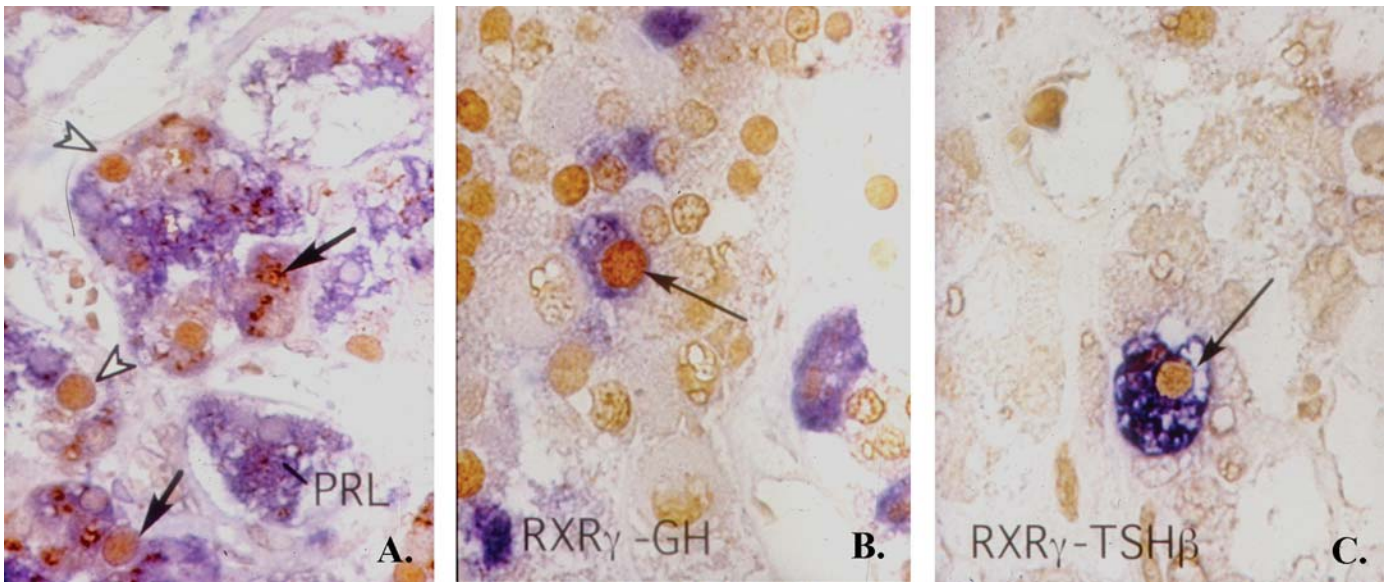
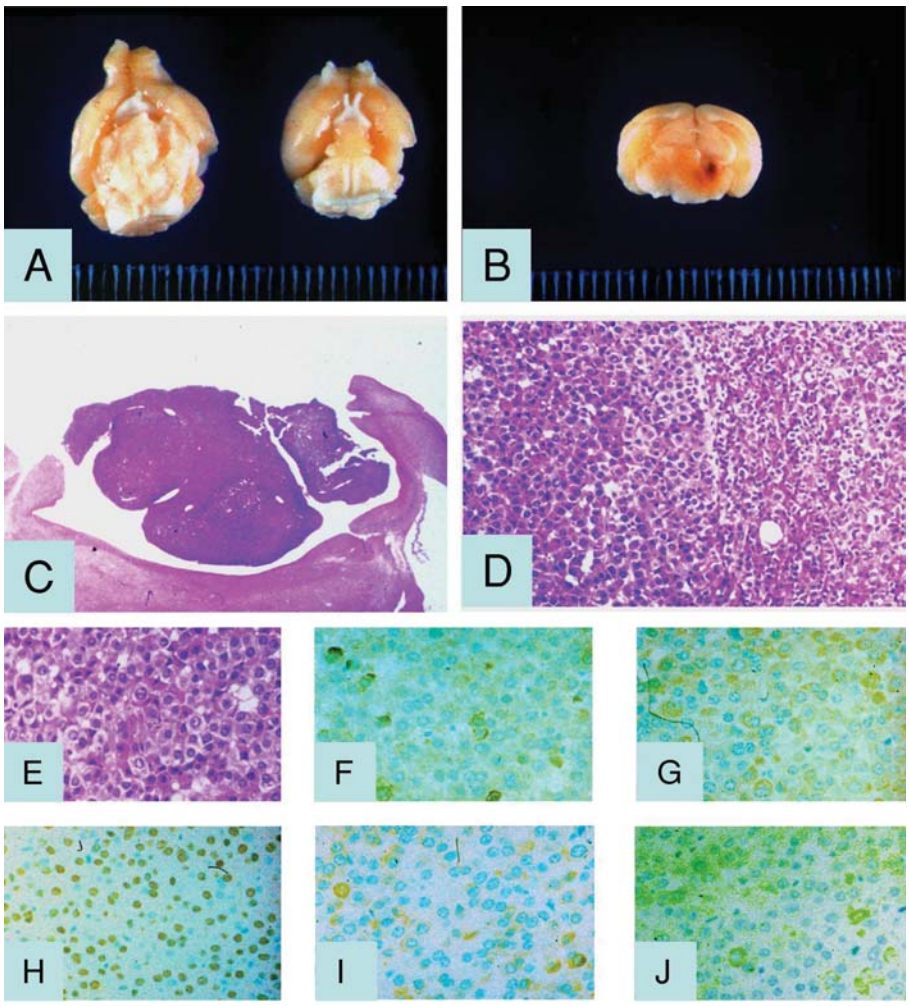


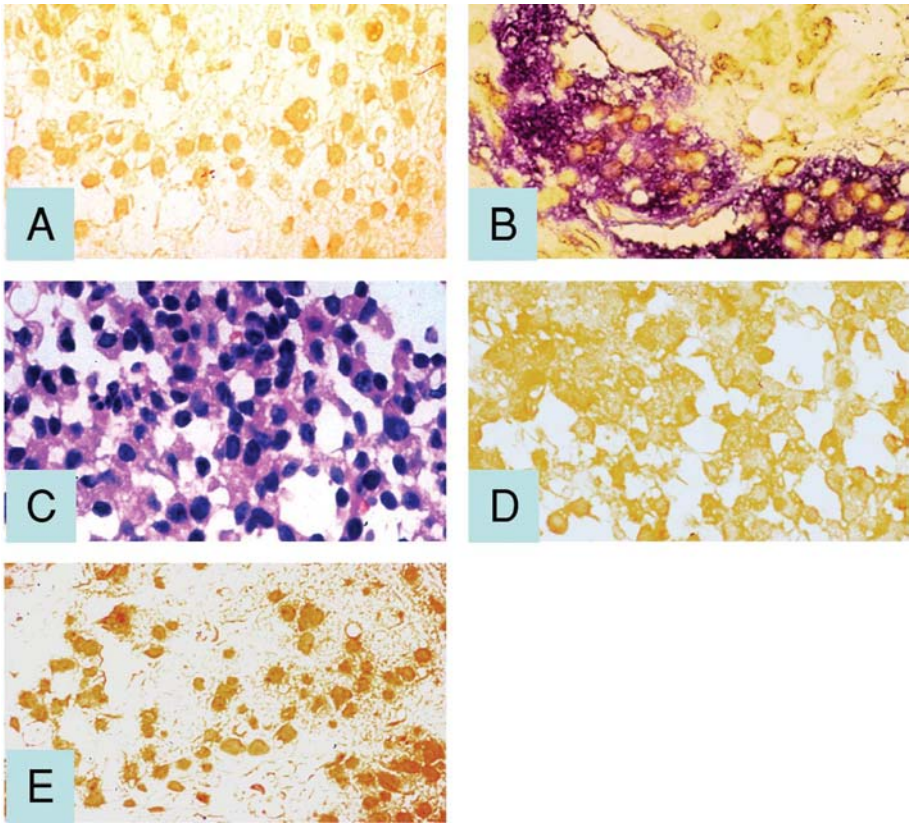
Color Plate 1, Fig. 1. (*see* full caption and discussion in Chapter 4, p. 76.)



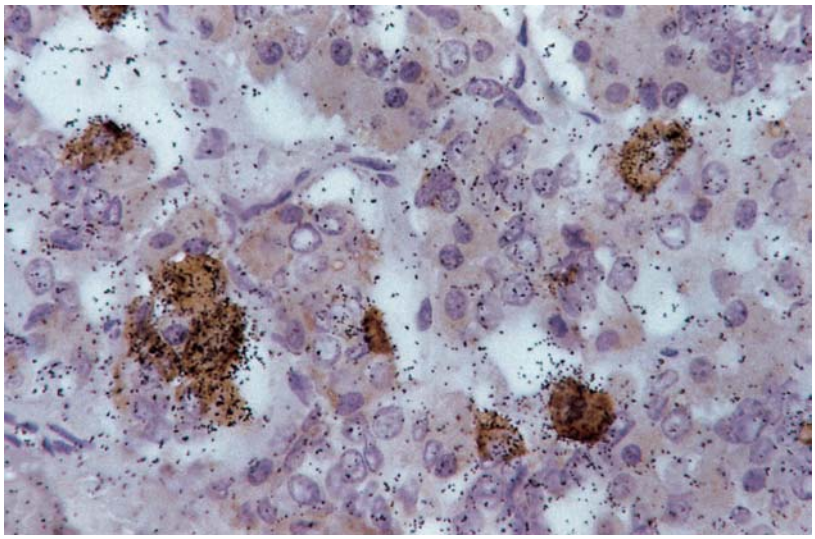
Color Plate 2, Fig. 8. (*see* full caption and discussion in Chapter 4, p. 82.)



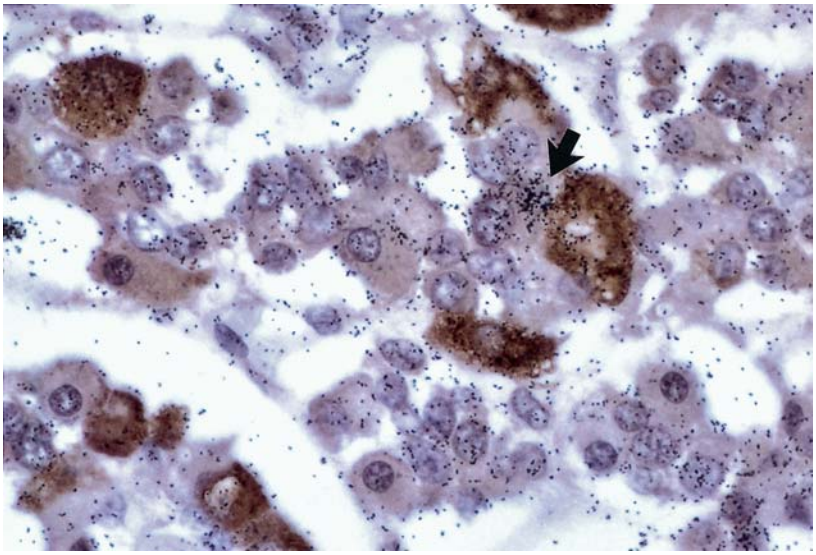
Color Plate 3, Fig. 9. (*see* full caption and discussion in Chapter 4, p. 83.)



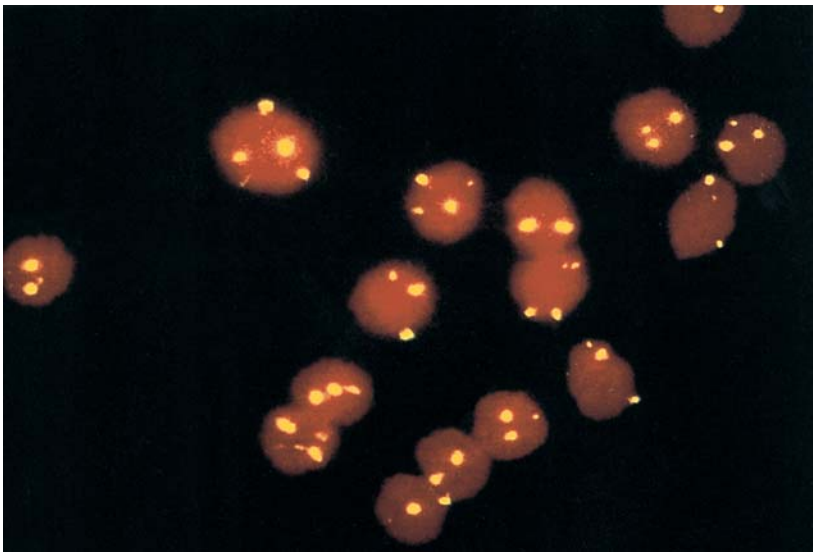
Color Plate 4, Fig. 11. (*see* full caption and discussion in Chapter 4, p. 85.)



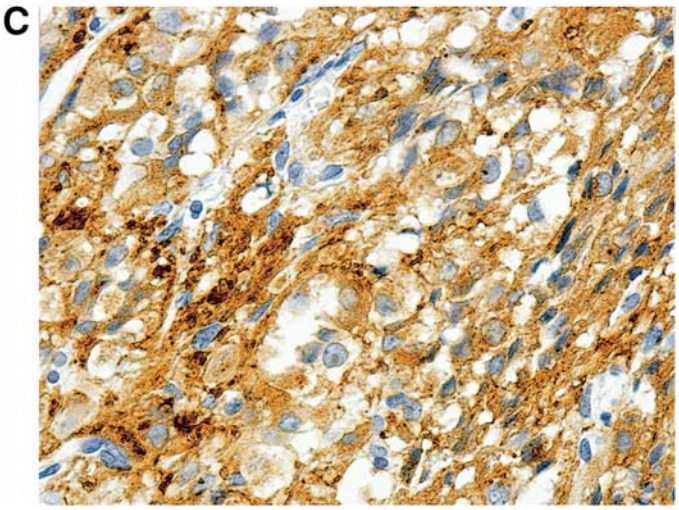
Color Plate 5, Fig. 3. (*see* full caption and discussion in Chapter 5, p. 91.)



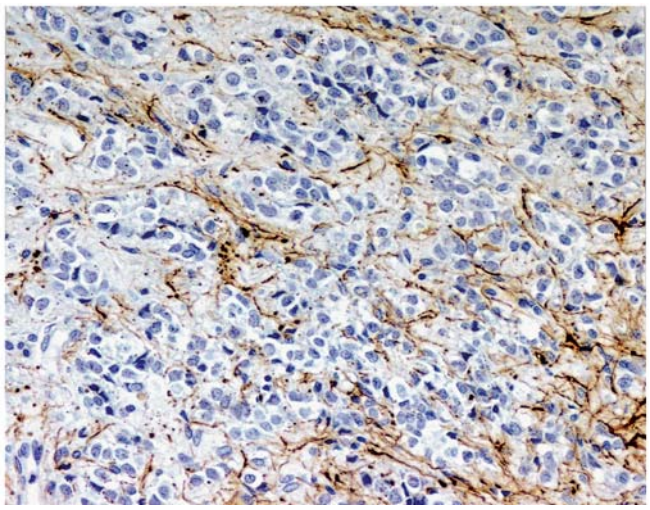
Color Plate 6, Fig. 4. (*see* full caption and discussion in Chapter 5, p. 92.)



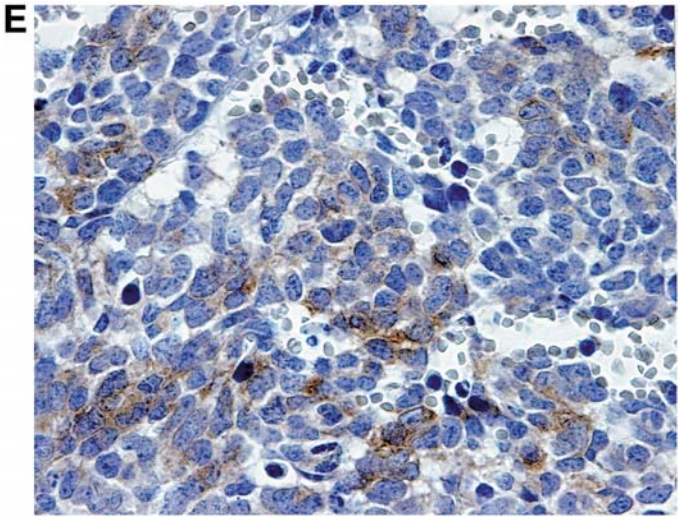
Color Plate 7, Fig. 5. (*see* full caption and discussion in Chapter 5, p. 92.)



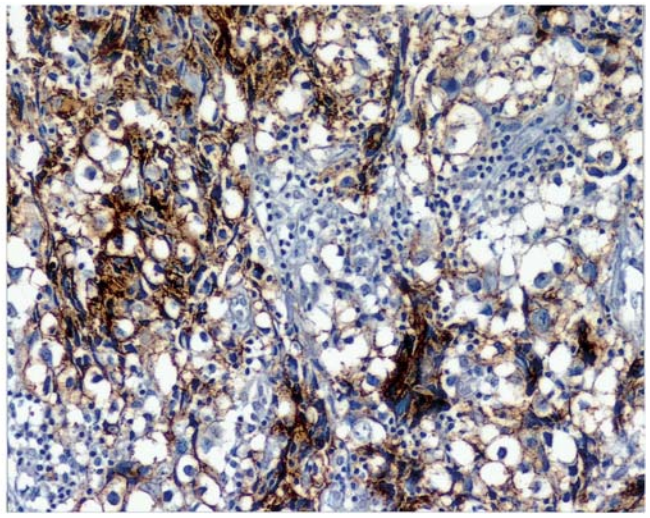
Color Plate 8, Fig. 1C. (*see* full caption and discussion in Chapter 6, p. 98.)



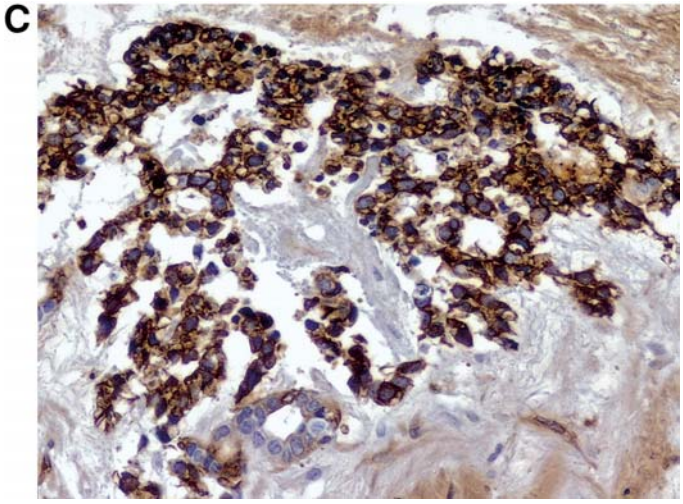
Color Plate 9, Fig. 1D. (*see* full caption and discussion in Chapter 6, p. 98.)



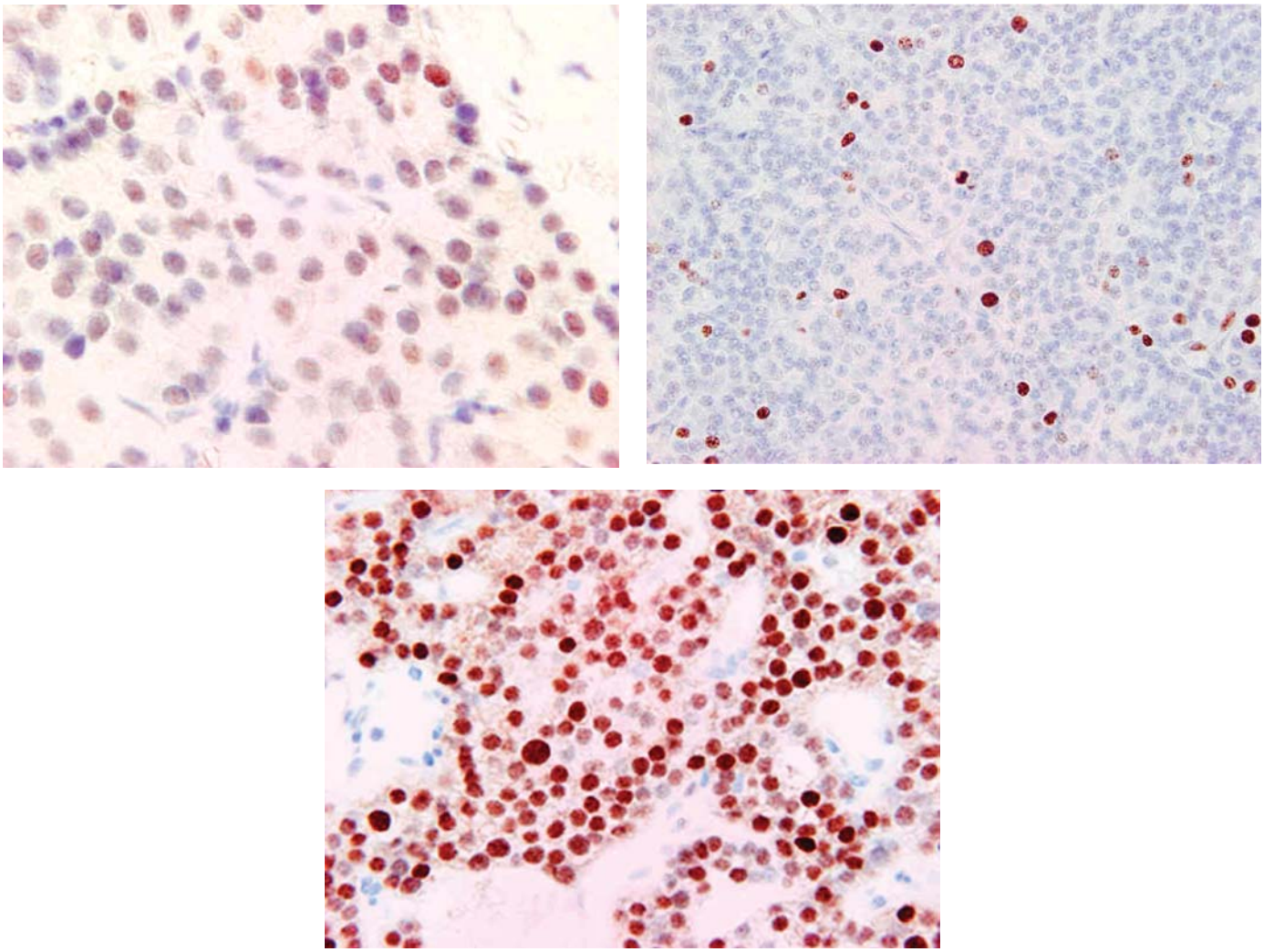
Color Plate 10, Fig. 3E. (*see* full caption and discussion in Chapter 6, p. 101.)



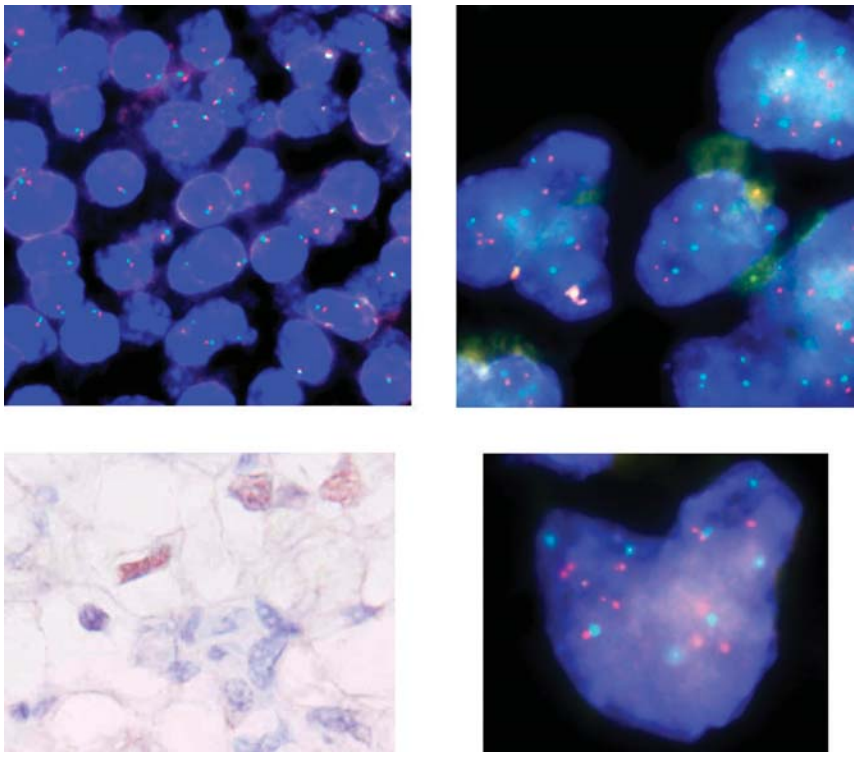
Color Plate 11, Fig. 6B. (*see* full caption and discussion in Chapter 6, p. 101.)



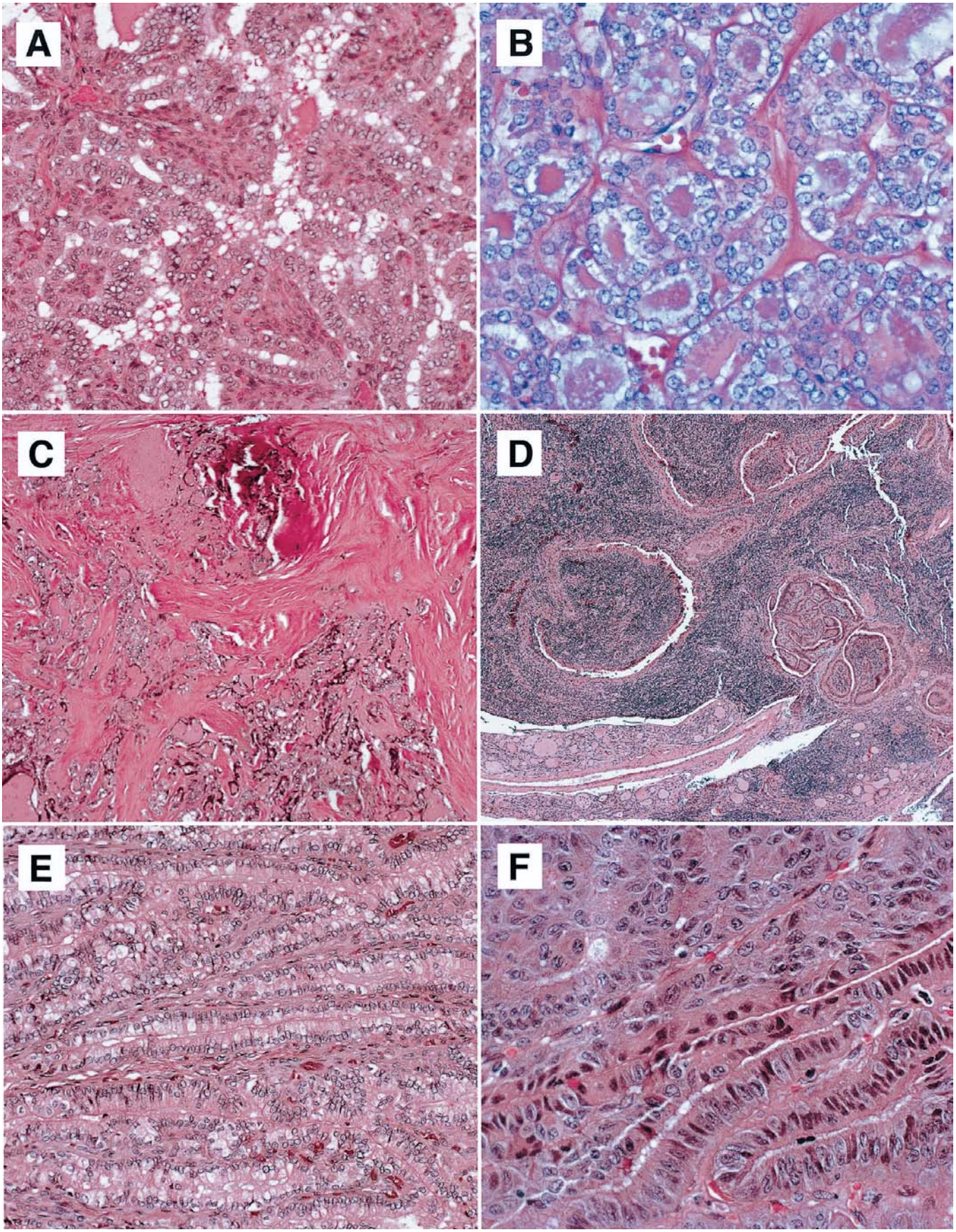
Color Plate 12, Fig. 7C. (*see* full caption and discussion in Chapter 6, p. 105.)



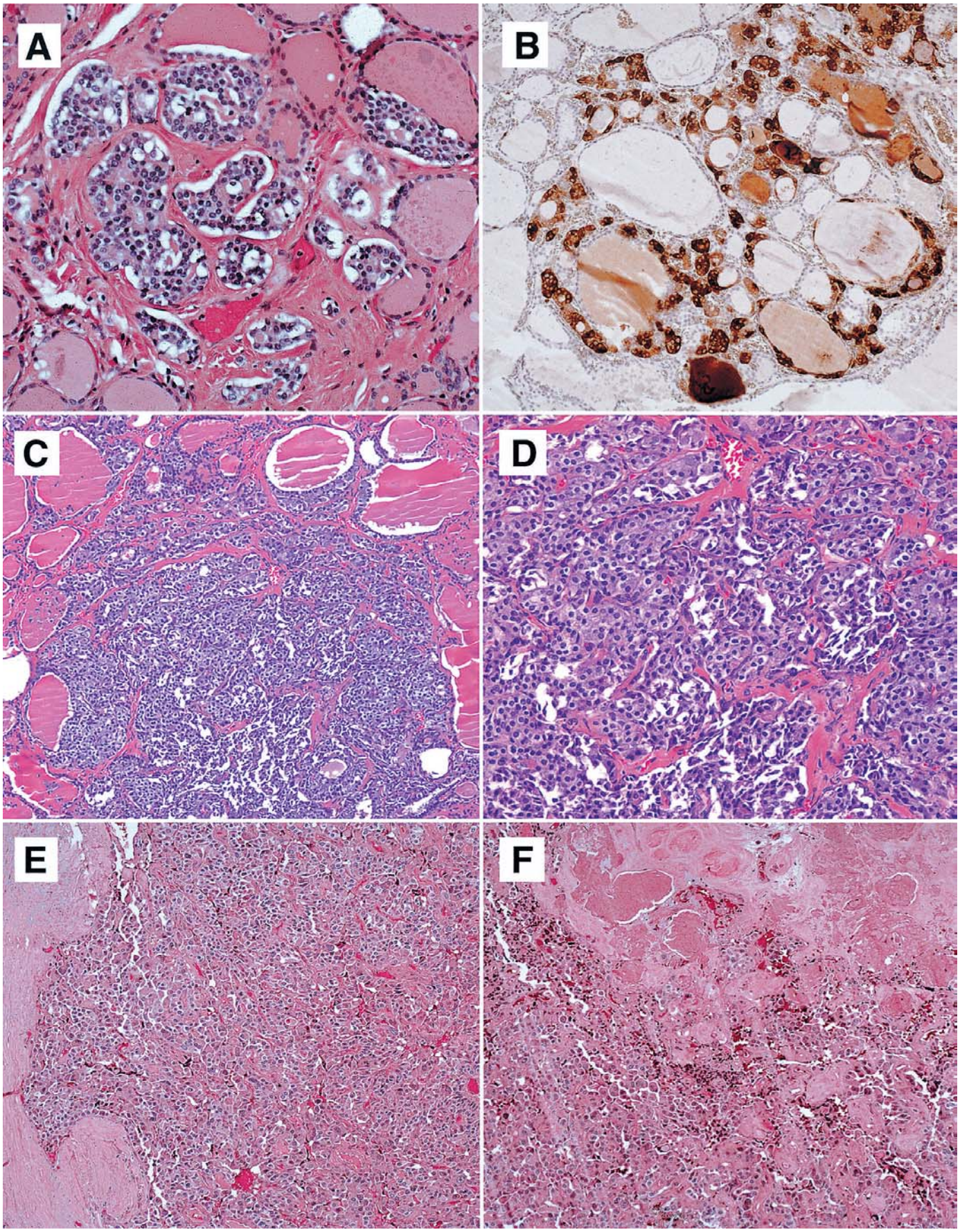
Color Plate 13, Fig. 7. (*see* full caption and discussion in Chapter 8, p. 141.)



Color Plate 14, Fig. 8. (*see* full caption and discussion in Chapter 8, p. 142.)



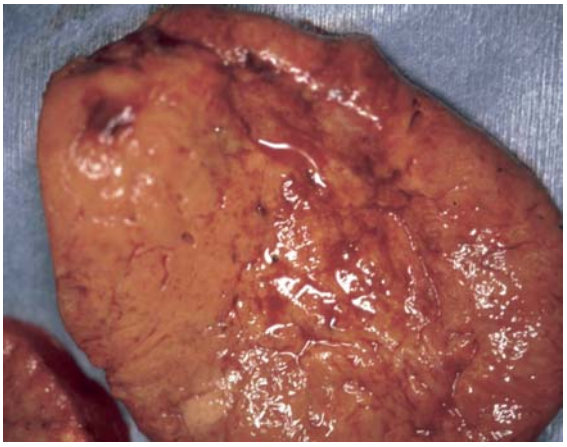
Color Plate 15, Fig. 11. (*see* full caption and discussion in Chapter 9, p. 172.)



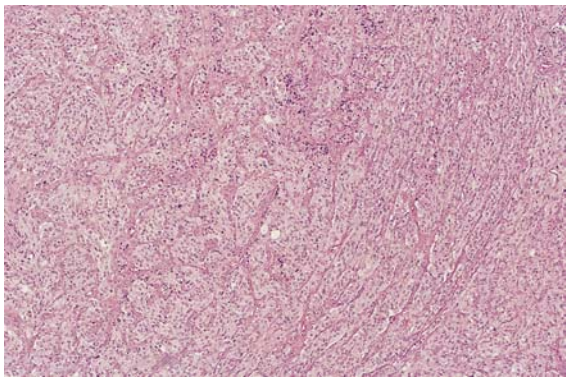
Color Plate 16, Fig. 14. (*see* full caption and discussion in Chapter 9, p. 180.)

Color Plate 17, Fig. 2. (*see* full caption and discussion in Chapter 11, p. 213.)

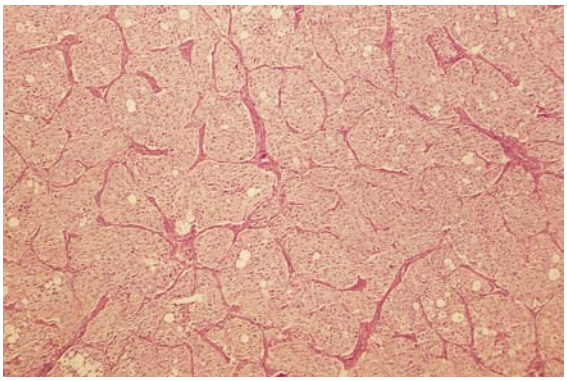
Color Plate 18, Fig. 9. (*see* full caption and discussion in Chapter 11, p. 218.)



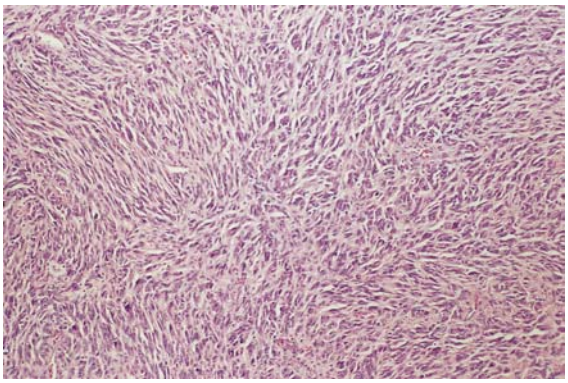
Color Plate 19, Fig. 10. (*see* full caption and discussion in Chapter 11, p. 220.)



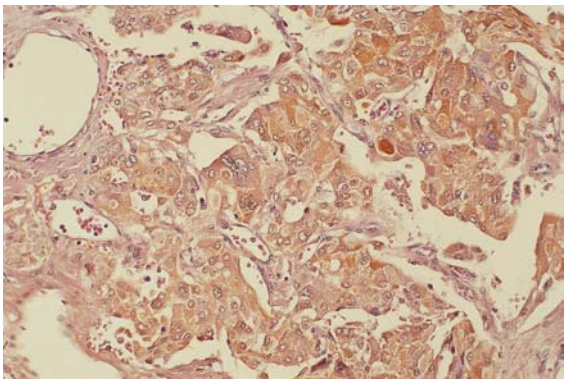
Color Plate 20, Fig. 5. (*see* full caption and discussion in Chapter 12, p. 231.)



Color Plate 21, Fig. 6. (*see* full caption and discussion in Chapter 12, p. 231.)



Color Plate 22, Fig. 7. (*see* full caption and discussion in Chapter 12, p. 231.)



Color Plate 23, Fig. 10. (*see* full caption and discussion in Chapter 12, p. 232.)

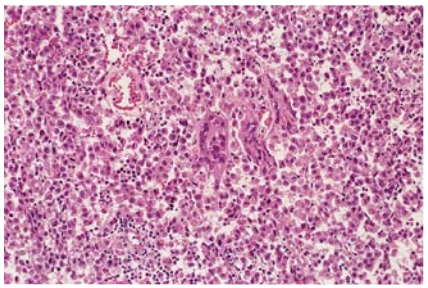


Figure 1

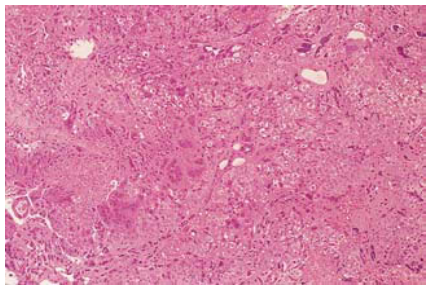


Figure 2

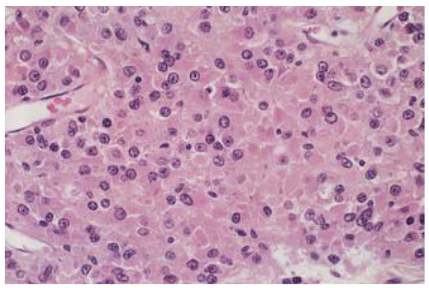


Figure 3

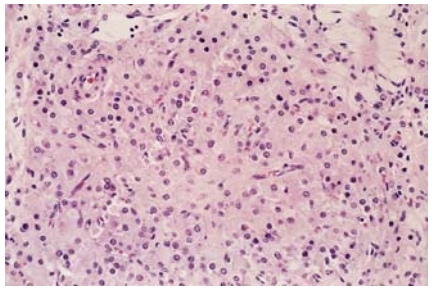


Figure 4

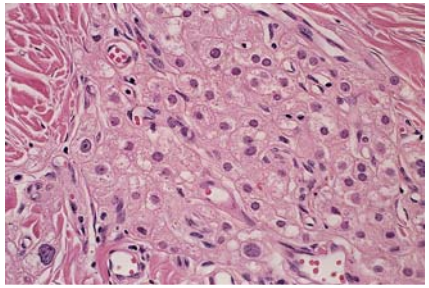


Figure 5

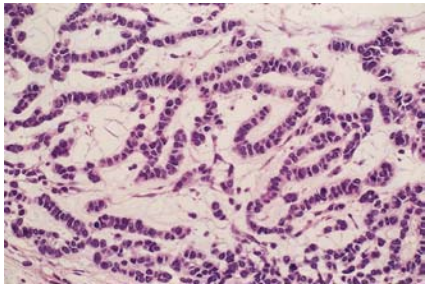


Figure 6

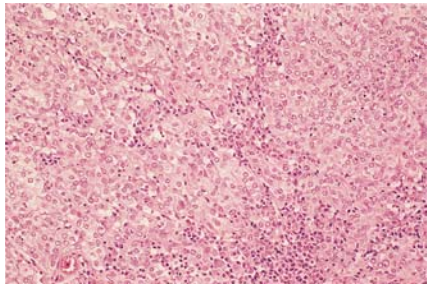


Figure 7

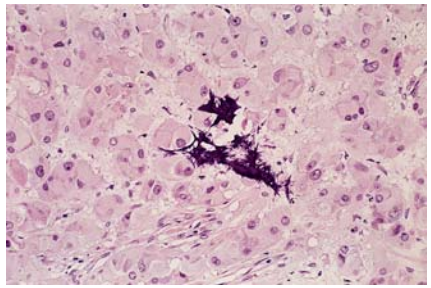


Figure 8

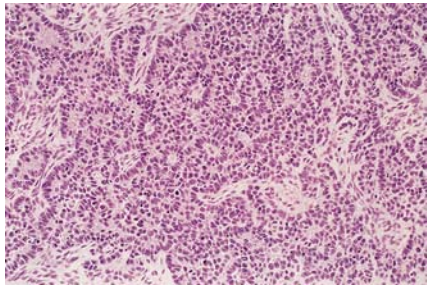


Figure 9

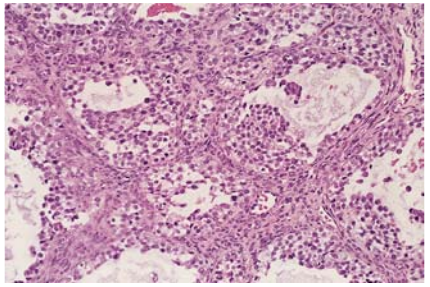


Figure 10

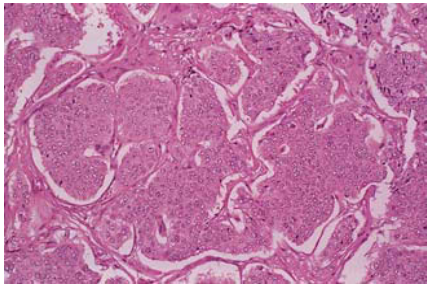


Figure 11

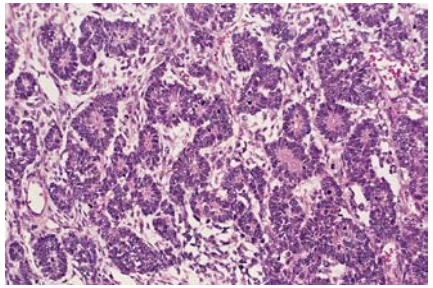


Figure 12

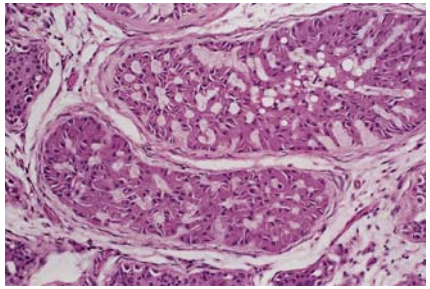
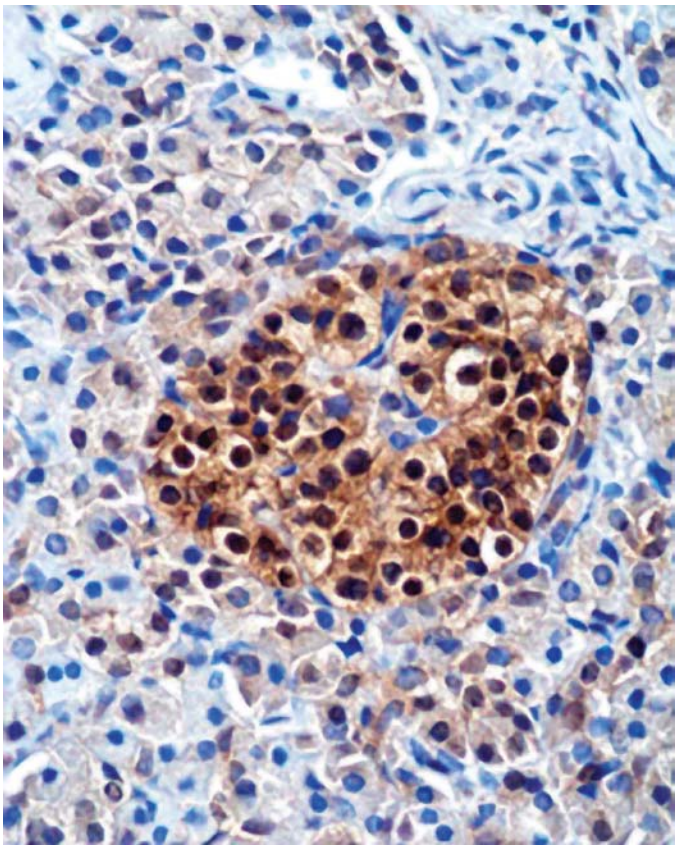
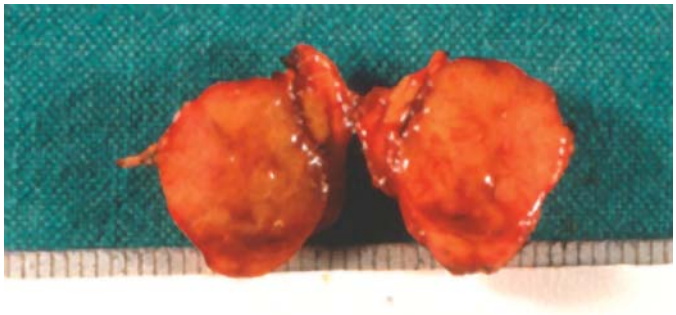


Figure 13

Color Plate 24, Figs. 1–13. (*see* full captions and discussions in Chapter 14, pp. 268–275.)

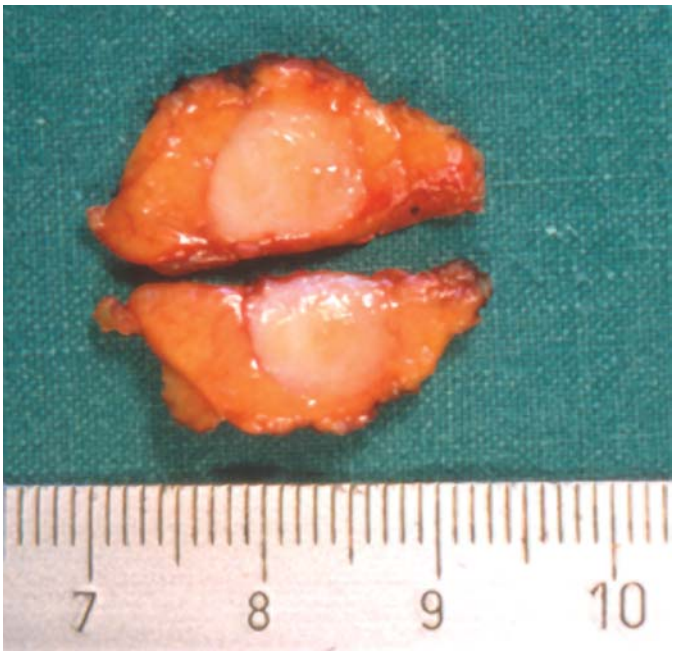


Color Plate 25, Fig. 4. (*see* full caption and discussion in Chapter 16, p. 295.)

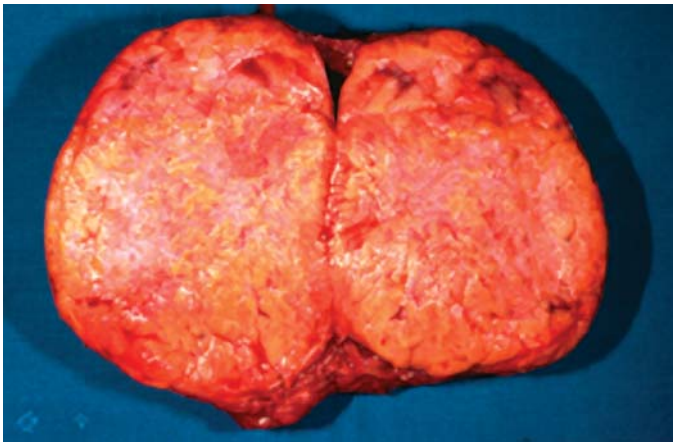


Color Plate 26, Fig. 10. (*see* full caption and discussion in Chapter 16, p. 303.)

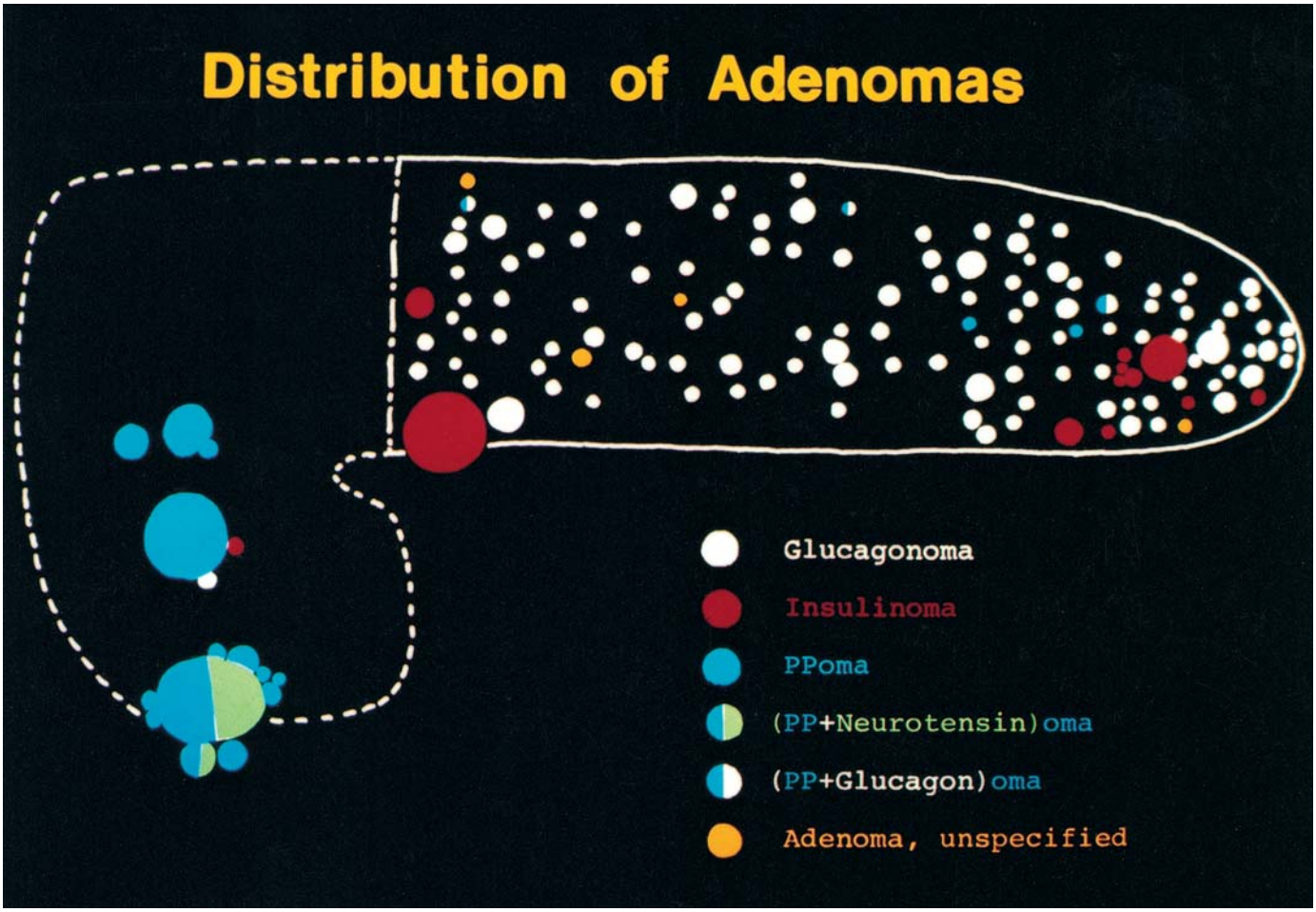
Color Plate 27, Fig. 25. (*see* full caption and discussion in Chapter 16, p. 312.)



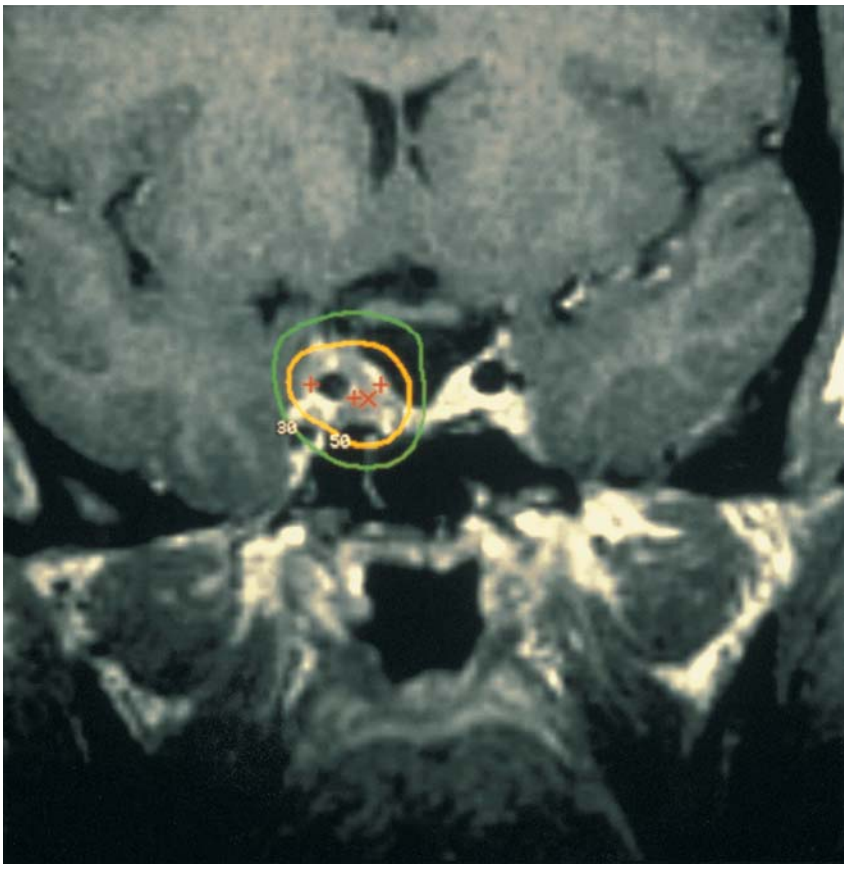
Color Plate 28, Fig. 27. (*see* full caption and discussion in Chapter 16, p. 314.)



Color Plate 29, Fig. 31. (*see* full caption and discussion in Chapter 16, p. 316.)



Color Plate 30, Fig. 2. (*see* full caption and discussion in Chapter 19, p. 361.)



Color Plate 31, Fig. 2. (*see* full caption and discussion in Chapter 23, p. 401.)

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9 Pathology of the Thyroid Gland

ASHRAF KHAN, MD, FRCPATH AND VÂNIA NOSÉ, MD, PHD

DEVELOPMENT AND EMBRYOLOGY

The thyroid gland develops from the larger median analage and the two lateral analagen. The medial analage, which forms the major portion of the thyroid, is derived from the floor of the foregut and the two lateral analagen are derived from the endoderm of the fourth and the fifth branchial pouches as the ultimobranchial bodies. The medial analage appears by d 24 as median endodermal diverticulum from the base of the tongue in the region of foramen cecum. The diverticulum descends down from the foramen cecum into the neck along the midline attached to the thyroglossal duct. It reaches its final position anterior to the trachea by about 7 wk; it then grows laterally, and becomes bilobed [1]. Early during the fifth week, the thyroglossal duct loses its lumen and shortly afterwards breaks into fragments [2]. However, the caudal end of the thyroglossal duct may persist in some embryos, and this constitutes the pyramidal process, which is present in about 75% of mature human thyroids [3]. The lateral thyroid analage becomes attached to the posterior surface of the thyroid during the fifth week and contributes up to 30% to the thyroid weight [2]. The causes of the fusion of the lateral and medial analage are unknown [2]. It is speculated that migration of the ultimobranchial body controls the growth of the medial analage, or that the growth of the medial analage laterally and caudally inhibits expansion of the ultimobranchial body [2]. The lateral thyroid analage is thought to give rise to the calcitonin producing C cells and the solid cell nests. It is believed that the C cells are derived from the neural crest; they migrate to the ultimobranchial body and are subsequently incorporated into the thyroid [4]. However, the existence of mixed follicular and C-cell tumors raises the possibility of the common stem cell origin for both follicular and C cells as is seen in the gastrointestinal tract [1].

The thyroid gland initially consists of a solid mass of endodermal cells, but small groups of epithelial cells are soon identified. The first follicles form from epithelial plates at the beginning of the eighth week and by the twelfth week the plates are entirely converted into follicles [2]. The development of the human fetal thyroid has been divided into three stages by LiVolsi to include precolloid stage (7–13 wk); the colloid stage (13–14 wk); and the follicular stage (after 14 wk) [5]. Evidence of thyroxin comes with the appearance of colloid and thyroxine (T4) and thyroid-stimulating hormone (TSH) are detectable in the circulation of human fetuses after 12 wk [2,3].

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NORMAL ANATOMY AND HISTOLOGY

The thyroid is normally located in the midportion of the neck anterior to the trachea and larynx, just below the cricoid cartilage attached by a loose connective tissue capsule. The recurrent laryngeal nerves lie in the groove between the lateral lobes and the trachea. The superior and inferior parathyroid glands are found close to the posterior surface of the gland or they may be located within the gland itself.

The thyroid gland consists of two lobes joined by an isthmus. In adults the two lobes measure approx 2–2.5 cm wide and 4–5 cm long [1]. In some glands a pyramidal lobe, derived from the distal portion of the thyroglossal duct, extends upwards from the isthmus. At birth the thyroid weighs 1–2 g and increases to 10–15 g at puberty [6]. In the adult the normal weight ranges from 15 to 35 g [1]. The weight varies with iodine intake, sex, age, and functional status of the gland. In addition there are geographic variations ranging from an upper limit of up to 42 g in a Portuguese study [7] to about 10–20 g in a North American population [8,9]. The mean weight is always higher in women, and the weight varies in size with the menstrual cycle [10]. In the elderly the weight may sometimes decrease to as little as 10 g [1].

On section, the thyroid gland has a brown cut surface and it is composed of multiple lobules separated by thin fibrous septa. Each lobule is made up of 20–40 follicles and is supplied by a single intralobular artery [1]. The thyroid gland contains three major types of epithelial cells: follicular cells, which line the follicles and secrete thyroxin and triiodothyronine (T3); C cells, which secrete calcitonin; and the solid cell nests (SCN), which are remnants of the ultimobranchial body. The follicles are filled with colloid, range in size from 50 to 500 μm (average 200 μm) in diameter, and are lined by cuboidal to low columnar epithelium. The cells lining the follicles have a basal nucleus and rest on a basement membrane composed of laminin and collagen IV [11]. The colloid includes thyroglobulin, which is a glycoprotein giving it a periodic acid-Schiff (PAS)-positive diastase-resistant staining characteristic. The amount of the colloid and the height of the follicular lining cells vary with the

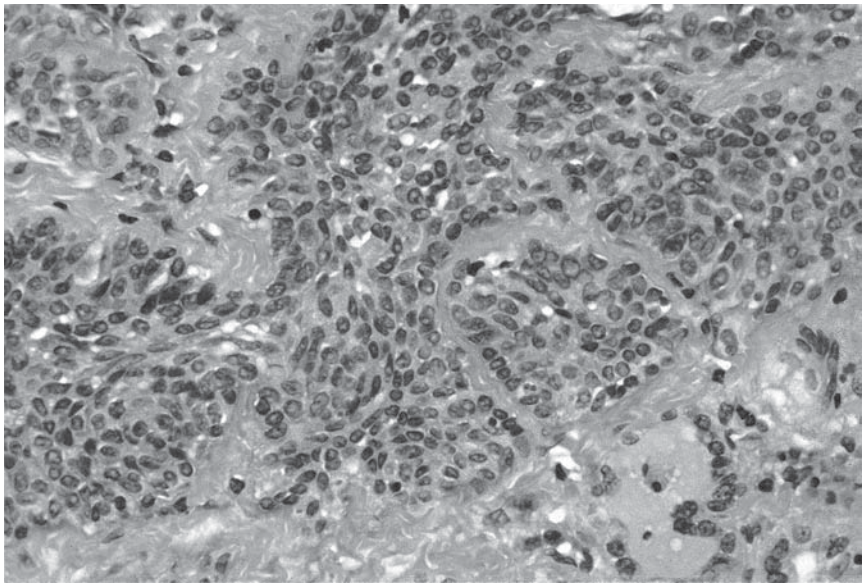

Figure 1 Solid cell nests in a thyroid gland (H&E stain).

functional status of the gland. In a hyperactive state the follicles are lined by tall cells and have less colloid. In addition to colloid the follicles contain birefringent calcium oxalate monohydrate crystals, the numbers of which may increase with age [1]. This finding may be useful at times to differentiate between thyroid follicles and parathyroid tissue at frozen section [12]. Intracytoplasmic fat within thyroid follicular cells may be detected in 50% of glands on oil red-O staining and this may increase with age [1]. Owing to the intimate development of thyroid with the mesodermal structures of the neck, fat, cartilage, and/or muscle may be found within the thyroid capsule [5]. For the same reason normal thyroid tissue may be found intermingled with the neck soft tissues including muscle, which should not be mistaken for metastatic carcinoma.

On immunohistochemistry the follicular cells stain positively with low-molecular-weight cytokeratin and with thyroglobulin, and may also coexpress vimentin [4]. *In situ* hybridization studies have revealed thyroglobulin mRNA within the follicular cells [4].

The C cells are intrafollicular and sometimes parafollicular in location. In normal glands they are not visualized on routine hematoxylin and eosin (H&E) staining. However, special histochemical stains such as Gremelius and immunohistochemical stains using pan-neuroendocrine antibodies such as chromogranin and synaptophysin and specific antibodies including calcitonin and calcitonin-gene-related peptide (CGRP) can identify them [4]. In addition, C cells may also stain positive for bombesin, somatostatin, gastrin-releasing peptide, low-molecular-weight cytokeratin, and carcinoembryonic antigen (CEA) on immunohistochemistry. On electron microscopy, the C cells have the characteristic neurosecretory granules; these granules are of two types, the larger and moderately electron dense type I granules measuring 280 nm, and the smaller 130 nm more electron dense type II granules. Both type I and II granules show calcitonin on immunoelectron microscopy [13]. On *in situ* hybridization, both calcitonin and CGRP mRNA can be localized in C cells [4].

The solid cell nests (SCN) can be seen in up to 60% of thyroid glands in the midportions of the lateral lobes [14]. They are solid irregular masses of epithelial cells measuring about 1 mm or less in maximum diameter and may be solitary or multiple, unilateral or bilateral. They are composed of polyhedral or oval cells with oval nuclei containing finely granular chromatin (Fig. 1); nuclear grooves may be seen [4]. SCN may sometimes show cystic change and a positive staining reaction with acid mucins [1]. On immunohistochemistry the SCN are positive for low-molecular-weight cytokeratin and CEA and show variable staining with calcitonin [1,4,15,16]. The latter finding supports the theory that SCN are derivatives of the ultimobranchial body. On electron microscopy, SCN demonstrate desmosomes, intermediate filaments, and intracytoplasmic microvacuolar structures [17]. In addition there are dendritic antigen-presenting cells present in the parafollicular thyroid stroma [18, 19]. Mast cells and T lymphocytes may also be seen around the follicles.

THYROID PHYSIOLOGY

The thyroid gland produces three hormones. T4 and T3 that are derived from the follicular cells and calcitonin is synthesized by the C cells. The production of T4 and T3 is controlled by a negative feedback mechanism via the hypothalamus–pituitary gland axis. The hypothalamus produces the thyrotropin-releasing factor (TRF), which in turn stimulates the production of TSH by the anterior pituitary [1]. The synthesis of T4 and T3 is dependent on the availability of exogenous iodine, which is essential for their synthesis. Deficient iodine intake, as discussed later, may lead to hypothyroidism and goiter. Furthermore, deficiencies in the activity of enzymes involved in thyroid hormone synthesis also lead to hypothyroidism, causing an increase in TSH that in turn produces hyperplasia and enlarge-

ment of the thyroid gland referred to as dyshormonogenetic goiter. Calcitonin plays a role in calcium homeostasis and causes a decrease in serum calcium levels.

DISORDERS OF THE THYROID GLAND

Disorders of the thyroid gland may present as hyperthyroidism or thyrotoxicosis, hypothyroidism, or enlargement of the gland (goiter) as a result of solitary or multiple nodules. The differential diagnosis of these thyroid disorders is outlined in Table 1.

DEVELOPMENTAL ABNORMALITIES OF THE THYROID

Abnormalities of Anatomical Development

Thyroid Agenesis and Hypoplasia Thyroid aplasia and hypoplasia are the cause of nongoiterous cretinism at birth. In some cases there may be aplasia of one lobe referred to as hemiagenesis; this is usually seen in the left lobe and is associated with normal thyroid function. In patients with di George syndrome, which is associated with third and fourth branchial pouch defects leading to developmental arrest of parathyroid and thymus, there may be arrest in C-cell development [1,4].

Ectopic Thyroid Tissue Ectopic thyroid tissue may be seen anywhere in the midline along the path of the thyroglossal duct. In such situations ectopic thyroid tissue may be present in addition to the normal thyroid, or ectopic tissue may be the only thyroid gland present. It is therefore important to search for normal thyroid before contemplating removal of the ectopic tissue to prevent hypothyroidism [1]. Locations of ectopic thyroid tissue include lingual, sublingual, suprahyoid, and mediastinum. Additional sites in which ectopic thyroid tissue has been found include the pericardium [4], heart, porta hepatis [20–22], gallbladder [23], inguinal region [1], vagina [24], sella turcica [25], trachea, and larynx. If sufficiently large, the tracheal and laryngeal ectopic tissue may produce respiratory symptoms [26,27].

Lingual thyroid resulting from complete arrest of the descent of the medial thyroid analage is rare, with a reported incidence of 1 in 4500 to 1 in 100,000 [1]. In most cases this is the only thyroid gland [1]. The lingual thyroid lacks a capsule and on histology thyroid follicles are seen mixed with the striated muscle fibers of the tongue [1]. While tumors have also been reported to occur in the lingual thyroid, pathologists must be careful in misinterpreting thyroid follicles intermingled with muscle as carcinoma. Diagnosis of carcinoma must be made only if there is marked desmoplasia or there are characteristic morphological features of papillary carcinoma [1]. Lingual thyroid may also be affected by thyroid disorders such as goiter and thyroiditis.

Ectopic thyroid tissue may also be found within the striated muscles and fibroadipose tissues of the neck [1,28] and may sometimes mimic submandibular gland swelling [29]. This usually is a result of a developmental defect due to a close association of thyroid and neck tissues during development. Sometimes benign appearing thyroid tissue may be found within the perithyroidal soft tissues of the neck later in life, which is a result of detachment of a portion of thyroid tissue from a large multinodular goiter [1,4]. It is important in these instances to differentiate this benign ectopic tissue or a detached portion of multinodular goiter from thyroid carcinoma.

Table 1
Differential Diagnosis of Thyroid Gland Disorders

HYPERTHYROIDISM
Diffuse toxic hyperplasia (Graves' disease)
Toxic multinodular goiter
Toxic adenoma
Subacute thyroiditis (de Quervain's thyroiditis)
Hyperfunctioning thyroid carcinoma (tumors derived from follicular epithelium)
Iatrogenic or drug-induced (excessive thyroid hormone supplement)
Struma ovarii (thyroid tissue associated with ovarian teratoma may cause excess ectopic hormone production)
Central hyperthyroidism; excess TSH
  TSH producing pituitary adenoma
  Pituitary resistance to thyroid hormone
Trophoblastic disease (hydatidiform mole and choriocarcinoma)

HYPOTHYROIDISM
1. Primary thyroid causes
  Insufficient thyroid parenchyma
    Hashimoto's thyroiditis *(most common cause in iodine sufficient areas)*
    Developmental abnormality (e.g., agenesis)
    Radiation injury (radioiodine, external radiation)
    Surgical ablation
    Interference with thyroid hormone synthesis
    Idiopathic primary hypothyroidism (possible immune blockade of TSH receptors)
    Biosynthetic defect: inborn errors of metabolism (e.g., dyshormonogenesis)
    Iodine deficiency
    Drugs (lithium, *p*-aminosalicylic acid, iodides, amiodarone, others)
2. Secondary hypothyroidism
  Pituitary destruction (tumor, infarcts, trauma)
  Isolated TSH deficiency
3. "Tertiary" hypothyroidism
  Hypothalamic destruction (reduced TRE delivery) due to tumor, granuloma, and trauma
4. Euthyroid "sick" syndrome: found in severe patients (sepsis, etc.) who appear chemically hypothyroid, but are not so.

DIFFUSE OR FOCAL ENLARGEMENT OF THE GLAND
Thyroiditides
  Hashimoto's thyroiditis
  Subacute granulomatous (de Quervain's) thyroiditis
  Riedel's disease (woody hard thyroid)
Hyperplasia/hypertrophy
  Diffuse toxic Goiter (Graves' disease)
  Diffuse nontoxic (simple) goiter
  Toxic multinodular goiter
  Nontoxic multinodular goiter
Neoplasia
  Benign tumors
    Follicular adenoma
  Malignant tumors
    Follicular cell derived
      Papillary thyroid-carcinoma (most common thyroid cancer)
      Follicular carcinoma
    Oncocytic (Hürthle cell) carcinoma
      Anaplastic carcinoma
    Parafollicular (C)-cell derived
      Medullary carcinoma
    Other
      Malignant lymphoma
      Metastatic carcinoma

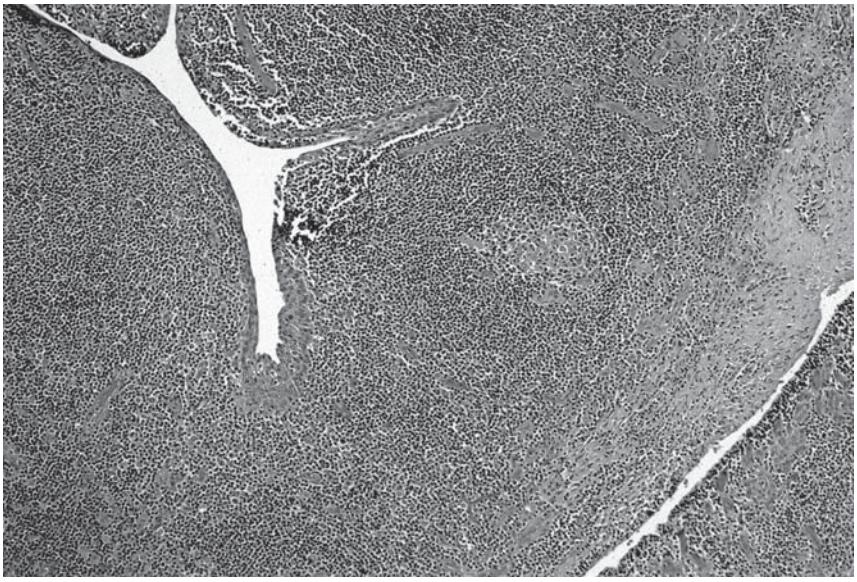

Figure 2 Thyroid gland with a lymphoepithelial cyst (branchial pouch remnant).

Lateral Aberrant Thyroid One of the most controversial and debated topics in thyroid heterotopia has been the finding of benign appearing thyroid follicles within the subcapsular sinuses of the cervical lymph nodes. In the past these had been referred to as lateral aberrant thyroid. It is believed that benign appearing thyroid follicles in lymph nodes medial to the jugular vein represent ectopic thyroid tissue, and a similar structure in nodes lateral to the jugular vein should be regarded as metastatic carcinoma [1,3,30–33]. However, the latter deposits may be seen in cases in which multiple serial sections of the thyroid gland failed to reveal any tumor [30,34]. It is possible that small microscopic thyroid carcinoma in these may have involuted, leaving a scar behind [30]. It has been suggested that normal thyroid tissue may be transported to the lymph nodes by way of lymphatics [32,33]. Architecture of the thyroid deposits has been suggested by some to be helpful in differentiation of benign thyroid tissue from metastatic deposits, but it has not been reproduced in some other studies [30]. In practice there is no definitive way at present to resolve this issue, but the finding of thyroid follicles within cervical lymph nodes should be investigated and a careful search for a primary tumor should be made in the thyroid by examining multiple sections. While morphology alone may not help to distinguish benign thyroid rests in lymph nodes from metastatic carcinoma, molecular diagnostic techniques to determine clonality have been suggested to establish the etiology of thyroid tissue within cervical lymph nodes [35].

Thyroglossal Duct Cysts Thyroglossal duct cysts (TDC) arises from the cystic dilatation of a persistent thyroglossal duct. It is located in the anterior midline of the neck, and although they are more common in children, they can present at any age and in either sex. On gross examination TDCs range in size from 1 to 4 cm in diameter, and fistula may develop secondary to infection, which may open into the pharynx or the skin. On microscopic examination the cyst is lined by respiratory and/or squamous epithelium. Thyroid tissue is seen in the cyst wall in approx 60% cases [1]. Recurrent infection may cause denudation of the lining epithelium and chronic inflammation and scarring in the cyst wall, leading to loss of the cystic architecture. However, a diagnosis of inflamed and fibrotic TDC may be made in the appropriate clinical setting even in the absence of the cyst lining [4]. Primary papillary thyroid carcinoma arising from the thyroid tissue within the thyroglossal duct cyst is a well-recognized complication [36–41]. Other primary thyroid tumors that have been reported to arise in thyroglossal duct cyst include Hürthle cell adenoma [42] and anaplastic carcinoma [43]. Although primary thyroid tumors can occur in the TDC, the possibility of metastases from a thyroid primary must be ruled out by careful evaluation of the thyroid gland. The criteria for the diagnosis of primary papillary carcinoma arising in TDC are: (1) histologic identification of TDC with cyst lining and thyroid tissue in the wall; (2) the presence of normal tissue adjacent to the tumor; and (3) failure of careful histopathologic evaluation of the thyroid to reveal a primary carcinoma [2].

Other developmental anomalies of the thyroid gland may be encountered in surgical pathology practice include the finding of a lymphoepithelial cyst similar in morphology to the branchial cleft cyst (Fig. 2). These are thought to be derived from the fourth and fifth branchial pouches which give rise to the ultimobranchial body that is involved in the development of the thyroid gland [44]. Evidence of thyroid development from the fourth branchial pouch is supported further by the finding of ectopic thyroid tissue in branchial cysts in the neck [45].

Hereditary Abnormalities Related to Thyroid Physiology

Genetic defects in any of the pathways that are involved in thyroid hormone synthesis may occur leading to dyshormonogenetic goiter. These are usually inherited as an autosomal recessive disorder and are present at birth. The clinical presentation of these disorders depends on the severity of the defect. A complete biochemical defect causes neonatal hypothyroidism (cretinism) and goiter at birth, while in partial defects goiter

may develop later in life. Some individuals may be euthyroid with elevated TSH [1]. Pendred's syndrome, which is characterized by goiter, congenital deafness, and positive perchlorate test is the most common form of dyshormonogenetic goiter [46]. The underlying molecular abnormality in Pendred's syndrome is mutations in the chloride/iodide transporter pendrin, which is encoded by the Pendred's syndrome (PDS) gene [47–50]. The exact role of pendrin is still unclear but it is thought to be involved in the transport of iodine, possibly at the apical membrane [47,50]. The abnormality in thyroid hormone synthesis in Pendred's syndrome is thought to be defective thyroid iodine organification, which is consistent with a positive perchlorate test [51–53] and is supported further by the finding of impaired thyroid organification in vitro by thyroid tissue taken from patients with Pendred's syndrome [51].

The histomorphology of the thyroid gland in dyshormonogenetic goiter may sometimes be challenging, and an overdiagnosis of malignancy in these cases represents a major pitfall. The elevated TSH causes the thyroid gland to be hyperplastic. On microscopic examination there are multiple hyperplastic nodules separated by fibrous septae, which may sometimes be associated with hemorrhage and calcification. The nodules are composed of extremely cellular follicles, which most often display a microfollicular or trabecular growth pattern with minimal colloid; focal areas of papillary hyperplasia may also be seen. The cells lining the follicles show marked cytologic atypia with evidence of multinucleation, hyperchromasia, nuclear enlargement, and mitotic activity, which may be mistaken for malignancy [1,30,46,53]. True malignant tumors occur in dyshormonogenetic goiters but are extremely rare. Papillary and follicular carcinoma have both been reported in dyshormonogenetic goiter; however, most commonly these tumors are follicular carcinoma, many of which may exhibit an aggressive phenotype including anaplastic transformation [47]. A diagnosis of malignancy in such a situation should be made only if there is unequivocal vascular invasion or evidence of distant metastasis [53,54].

NON-NEOPLASTIC THYROID DISORDERS

Graves' Disease

Definition Graves' disease is a form of thyroid autoimmune disease characterized by diffuse hyperplasia of the thyroid gland, thyrotoxicosis due to excessive thyroid hormone synthesis, and the presence of thyroid-associated autoantibodies in the serum.

Historical Background As early as the fifth century BC the ancient Greeks were known to describe the combination of goiter, exophthalmos, and palpitations which is what is now recognized as a constellation of symptoms seen in patients with Graves' disease [30]. In 1786, Parry [3] was the first to recognize and describe a group of patients with a combination of symptoms including rapid heart beat, goiter, and sometimes exophthalmos. These findings were, however, published in 1825 after Parry's death by his son in an obscure book [55]. Later Robert Graves, in 1835, and Carl Basedow, in 1840, also described a group of patients with symptoms similar to those described by Parry [55]. As the continental Europeans were not aware of Graves' description, in continental Europe this disease became to be known as Basedow's disease and some in Europe still use this term rather than Graves' disease [55]. Both Parry and Graves thought that these symptoms were a result of a cardiac disease, and even after Basedow's report the goiter was not considered of much importance [55]. However, after the description by Charcot of additional cases with the above symptoms associated with nervousness, the cardiac origin gave way to a neurologic etiology around 1860, a belief that was dominant for the rest of the 19th century [55]. By the late 19th century, when surgeons were able to remove goiters, it was observed that removal of goiters improved nervousness in patients who survived surgery. This fact, supported by the observation in the 1890s that too much thyroid extract led to similar nervousness and weight loss, gave way to the thyroid origin of what is now universally known as Graves' disease [55].

Clinical Features Graves' disease is seen mostly in women, with a female-to-male ratio of 8:1; the most common age at presentation is in the third or the fourth decade [4]. Patients have diffuse enlargement of the thyroid (goiter), thyrotoxicosis with associated ocular and skin changes, and cardiac manifestations. The most common symptoms and signs include nervousness, excessive sweating, heat intolerance, palpitations, fatigue, tachycardia, muscle wasting, weight loss, diffuse goiter, tremors, and eye changes [4].

Pathogenesis Graves' disease is an autoimmune disease characterized by the presence of both B and T lymphocytes that are sensitized to thyroid autoantigens. The primary thyroid autoantigen in Graves' disease is the TSH receptor [56]. Other secondary autoantigens involved in Graves' disease include thyroid peroxidase, thyroglobulin, and the sodium/iodide cotransporter [56,57]. Two types of TSH receptor antibodies have been identified; these are the TSH receptor-stimulating antibody and the TSH receptor-blocking antibody [56]. Both of these antibodies have been found in patients with Graves' disease, and the degree of thyroid stimulation in these patients is dependent on the relative concentration and bioactivity of the different autoantibodies [58]. The TSH receptor-stimulating antibody is specific to Graves' disease, whereas other autoantibodies such as the antithyroglobulin, antiperoxidase antibodies may be seen in patients with autoimmune thyroiditis and sometimes in normal individuals, in addition to patients with Graves' disease [56]. The TSH receptor-stimulating antibody functions like TSH, causing activation of the adenyl cyclase–cAMP and protein kinase C–phosphoinositide signal transduction pathways [59], leading to increased synthesis and release of thyroid hormone and hyperplasia of thyroid follicular cells.

T cells, predominantly T helper (CD4) cells of both Th1 and Th2 subtypes, constitute the majority of the intrathyroidal lymphocytes in Graves' disease. Other cell types, which are in the minority, include T suppressor (CD8) lymphocytes, B lymphocytes, and plasma cells. Furthermore, it has been shown that when intrathyroidal lymphocytes from Graves' disease are grown in vitro, the T cells are primarily Th2 cells with considerable T helper cell activity [60,61]. What initiates the autoimmune reaction in Graves' disease in not entirely clear. Possible mechanisms include molecular mimicry or cross-reactivity resulting from structural similarity between infectious agents and thyroid proteins, such as the similarity between *Yersinia enterocolitica* and the TSH receptor [62]. There is no evidence,

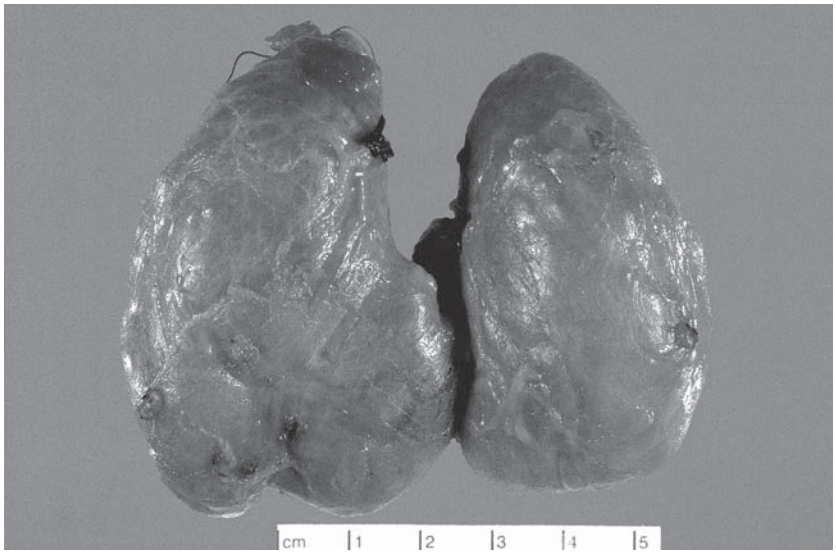

Figure 3 Gross appearance of thyroid gland in Graves' disease.

however, that either *Yersinia* or other viral infection causes Graves' disease. Other mechanisms suggested for the initiation of the immune reaction in Graves' disease include direct induction major histocompatibility complex (MHC) class II molecules such as HLA-DR on the surface of thyrocytes as a result of viral infection. Thus, the thyroid follicular cells may act as antigen-presenting cells themselves, leading to the production of thyroid autoantibodies [63]. Risk factors in the causation of Graves' disease include genetic susceptibility such as HLA-B8, HLA-DR3 in Caucasians; HLA-DR5 in Japanese; HLA-DR9 in Chinese; HL-b13 and DR5/8 in Koreans [64]; infections, stress, sex steroids, pregnancy and the postpartum period, cigarette smoking, and iodine [56].

Pathology of Graves' Disease The gross and microscopic appearance of the thyroid gland in Graves' disease varies with the amount and effectiveness of preoperative medical treatment. In general, the thyroid gland shows diffuse and symmetrical enlargement with a smooth surface (Fig. 3). The gland may weigh up to 150 g, is softer in consistency than the normal thyroid, and sectioning reveals a fleshy red-brown cut surface. Histological examination shows follicles of varying size lined by tall columnar cells with a decrease in the amount of colloid and evidence of scalloping of the colloid at the periphery of the follicle. The lining follicle cells may show piling up with formation of pseudopapillary structures. This latter feature together with the presence sometimes of optically nuclei and rarely foci of calcifications mimicking psammoma bodies should not be mistaken for papillary carcinoma. The important distinguishing feature in Graves' disease is the diffuse presence of these changes throughout the gland; the presence of round, basally placed nuclei; and the absence of other nuclear changes of papillary carcinoma [30]. Additional histological features in Graves' disease include a variable degree of lymphocytic infiltrate in the stroma and interstitial lymphoid cells that are predominantly of the T-helper (CD4) subtype that aid in the production of antibodies by the B cells [65,66]. Preoperative medical treatment may alter the characteristic histologic picture in Graves' disease. This may lead to larger follicles that are filled with colloid and follicular atrophy with Hürthle cell metaplasia mimicking Hashimoto's thyroiditis [67,68]. Preoperative treatment with radioactive iodine may cause follicular disruption and atrophy with associated Hürthle cell metaplasis, fibrosis, and cellular and nuclear pleomorphism [1].

Thyroid Nodules and Cancer in Graves' Disease Thyroid nodules are a common clinical problem in the general population, but the prevalence of palpable thyroid nodules in Graves' disease is increased by more than threefold compared to the general population [69]. Whereas the prevalence of palpable thyroid nodule is 3.2–4.7% in iodine-sufficient areas [70], in one large multiinstitution study the prevalence of palpable thyroid nodules in patients with hyperthyroidism was 15.8% [71]. Other studies have reported similar results [68,69,72]. On review of multiple studies, the prevalence of cancer in Graves' disease has been reported to range from 0 to 9.8%, while in Graves' disease patients with palpable nodules the prevalence increased, ranging from 5.8% to 45.8% [69]. In a recent study that included 325 patients with Graves' disease, Stocker et al. found papillary thyroid carcinoma in 1.85% of all these patients, in 15.2% of these patients with cold nodules on scintiscan; in 25% of these patients with palpable nodules, and in 27.3% of those undergoing surgery [73]. Thus it seems that thyroid scintigraphy is an important preliminary investigation in the evaluation of Graves' disease patients and the high prevalence of thyroid cancer in those patients with cold nodules and palpable nodules warrants further diagnostic evaluation including a fine needle aspiration biopsy. Thyroid cancer arising in the background of Graves' disease is thought to be of a more aggressive phenotype compared to that in euthyroid patients. The patients may present with nodal and distant metastases, bilateral and multicentric tumors with invasive growth [74–77]. This, however, has not been supported by some other studies [72,78–80]. It is interesting to hypothesize that the presence of anti-TSH receptor-stimulating antibodies may cause stimulation of thyroid cancer cells and early metastatic growth in the same way

Table 2
Classification of Thyroiditis

I.	Infectious thyroiditis
	Bacterial
	Fungal
	Mycobacterial
	Parasitic
	Viral
II.	Autoimmune inflammation
	Hashimoto's thyroiditis
	Postpartum thyroiditis
III.	Uncertain etiology, possibly autoimmune
	Riedel's thyroiditis
	Atypical subacute (silent) thyroiditis
	Focal lymphocytic thyroiditis
IV.	Nonimmune
	Subacute (de Queverain's) thyroiditis

as TSH stimulates growth of tumor cells expressing TSH receptor [69].

Thyroiditis Thyroiditis can be caused by multiple factors among which autoimmune inflammation appears to be the most common etiological insult. A classification based on etiology is proposed in Table 2. From this table it seems that after infection has been ruled out (which at any rate is a rare cause of thyroiditis), most other types of thyroiditis have either a definitive autoimmune etiology or are possibly autoimmune in nature. It is possible that these noninfectious forms of thyroiditis other than subacute and palpation thyroiditis may be part of a spectrum of autoimmune thyroiditis with different precipitating factors and manifestations.

Infectious Thyroiditis The thyroid gland appears to be relatively resistant to infection, making infectious thyroiditis a rare occurrence. Protective mechanisms that have been suggested for making the thyroid resistant to infections include a rich blood supply to the gland and lymphatic drainage from the gland, a high glandular iodine content of the gland which may act as a bactericidal agent, and a fibrous capsule around the thyroid and its anatomic separation from other neck structures by fascial planes [81]. The most common predisposing factors for thyroid infections are preexisting thyroid disease including multinodular goiter, Hashimoto's thyroiditis, and thyroid cancer [81–83]. Different modes of infection to the thyroid include spread from a primary focus via the bloodstream or lymphatics; direct spread from adjoining neck structures such as infected thyroglossal duct cyst, pharynx, or tonsil; and following neck trauma [5,81]. Direct inoculation following thyroid surgery is extremely rare [81].

ETIOLOGY OF THYROID INFECTIONS Acute bacterial thyroiditis is the most common cause of infectious thyroiditis [84–86]. The most common clinical presentation includes thyroid pain, fever, tenderness, and local mechanical compression leading to dysphagia and dysphonia [81]. The pathogens that have been most commonly implicated include *Staphylococcus aureus* and *Streptococcus pyogenes* in adults and α- and β-hemolytic Streptococcus and a variety of anaerobes in children [81]. Following bacterial infections, fungal infection is the next most common cause of infectious thyroiditis [86]. *Aspergillus* species is the most commonly documented fungal infection in the thyroid, which most often occurs in the setting of an immunosuppressive state such as glucocorticoid therapy, and in the presence of leukemia and lymphoma [81]. Other causes of fungal thyroiditis that have been reported include *Candida* [87], *Histoplasma* [88], cryptococcus [4], Coccidiodes [89], and *Nocardia* [90]. Mycobacterial infection of the thyroid is very rare and most often presents in the form of disseminated or miliary tuberculosis [81]. Infection with atypical mycobacterium such as *Mycobacterium avium intracellulare* has been reported in patients with acquired immunodeficiency syndrome (AIDS) [91] and also in an immunocompetent patient with Hashimoto's thyroiditis [92]. Parasites such as *Echinococcus granulosus*, *Strongyloides stercoralis*, and *Taenia solium*, the latter causing cysticercosis, have been reported to cause thyroiditis. Viral infections such as rubella and cytomegalovirus have been reported to cause thyroiditis [4]. Infection with cytomegalovirus (CMV) usually occurs in the setting of an immunosuppressive state. Opportunistic infectious agents that have been isolated from the thyroid in patients with AIDS include CMV [93,94], *M. avium intracellulare* [91], and *Pneumocystis carinii* [93]. These usually occur in the setting of widely disseminated infections and CMV and *P. carinii* both may be asymptomatic and isolated at autopsy. However, *P. carinii* may present with painless thyroid nodule, cold on radionuclide scan, increasing in size, and associated with hypothyroidism [81].

PATHOLOGY OF INFECTIOUS THYROIDITIS Bacterial and fungal infections cause acute suppurative thyroiditis, which on gross examination may show a normal or slightly enlarged gland often with associated with focal necrotic areas on sectioning [4]. Histological examination shows acute suppurative inflammation associated with necrotic foci with microabscess. A careful search should be performed for microorganisms and special stains used to look for bacteria and fungi if an infectious etiology is suspected. Fungal infections may sometimes produce a granulomatous inflammation. Mycobacterial infection causes a granulomatous thyroiditis; these epithelioid granulomas may be non-necrotizing or sometimes associated with caseous necrosis. Patients with an immunosuppressive disorder may not be able to mount a granulomatous reaction. Special stains for acid-fast bacilli should be performed when mycobacterial infection is suspected. The causes of granulomas in thyroid are listed in Table 3. Infection with CMV will show typical intracellular inclusions as are seen in other organs. *Pneumocystis carinii* are identified on methamine silver stain, which should always be performed in cases suspected to have *P. carinii*, especially patients with AIDS.

Hashimoto's Thyroiditis

HISTORICAL BACKGROUND In 1912, H. Hashimoto [95] described four cases goiter for which he coined the term "struma lymphomatosa" (lymphomatous goiter). All four of Hashimoto's cases were females and showed histological changes similar to those seen in autoimmune thyroiditis, which have come to be known as Hashimoto's thyroiditis. The autoimmune nature of this form of thyroiditis was established in 1956, when Roitt et al. reported

Table 3
Conditions Associated with Granulomas in Thyroid

1. Subacute (de Queverain's) thyroiditis
2. Palpation thyroiditis
3. Mycobacterial infections
4. Fungal infections
5. Sarcoidosis
6. Histiocytic reaction associated with hemorrhage in goiter and tumors
7. Granulomatous vasculitis
8. Foreign body reaction
9. Postooperative necrotizing granulomas

autoantibodies against thyroglobulin in patients with Hashimoto's thyroiditis [96]. One year later Trotter et al. in 1957 [97] identified a second antigen in the microsomal fraction of thyroid homogenates, which proved to be thyroid peroxidase (TPO).

CLINICAL FEATURES Hashimoto's thyroiditis most frequently affects middle-aged women and is also the most common cause of sporadic goiter in children. Patients may present with hypothyroidism, goiter, or both. Widespread use of thyroid function tests have also identified many cases of Hashimoto's thyroiditis with subclinical hypothyroidism characterized with positive anti-TPO and anti-thyroglobulin antibodies in the serum associated with high TSH and normal T4 [98]. The goiter when present is firm and often lobulated, which may be mistaken for a multinodular goiter or carcinoma. Hashimoto's thyroiditis presenting as goiterous hypothyroidism has been found to be associated with HLA-D3 and HLA-D5 [99]. It may also coexist with other autoimmune diseases including pernicious anemia, diabetes mellitus, Sjögren's syndrome, chronic hepatitis, adrenal insufficiency, and Graves' disease. There appears to be a familial predisposition for the development of Hashimoto's thyroiditis; up to 5% first-degree relatives of patients with Hashimoto's thyroiditis may have positive antithyroid antibodies in their serum [99,100,101]. There is also a high prevalence of autoimmune thyroid disease in patients with Down's syndrome, familial Alzheimer's disease, and Turner's syndrome [102–106].

PATHOGENESIS OF HASHIMOTO'S THYROIDITIS The pathogenesis of Hashimoto's thyroiditis is summarized in Fig. 4. Hashimoto's thyroiditis is a thyroid-specific autoimmune disorder in which both humoral and T-cell-mediated cellular immune mechanisms play a role. The inflammatory process is initiated by the activation of thyroid-specific CD4 or T helper cells [107]. The cause of this T helper cell activation is not entirely clear; both viral and bacterial infections have been implicated [108–110], but there are no conclusive data to support this. Another hypothesis implies that thyrocytes express HLA-DR antigen and become antigen-presenting cells, thereby exposing the thyroid cellular proteins to the CD4 T helper cells, which initiates the formation of autoantibodies against the thyroid autoantigens [111–114]. Once the T helper cells are activated, they stimulate the B cells to produce antibodies [115] against thyroid antigens such as thyroglobulin, thyroid peroxidase, TSH receptor, and thyroid microsomal antigen leading to antibody-mediated thyroid cell injury. In addition to this pathway of thyroid cell injury, an alternative pathway involving cytotoxic T (CD8) cells and apoptosis has been more recently proposed [116–125]. It has been shown that a significant population of intrathyroidal lymphocytes in Hashimoto's thyroiditis are of CD8 phenotype having a cytotoxic/suppressor activity (reviewed in 48,126,127). Some recent studies have shown that follicular cells from tissue samples of Hashimoto's thyroiditis exhibit strong staining for the death receptor Fas and its ligand FasL, together with a high apoptotic rate as compared normal controls [128,129]. Bcl-2 inhibits apoptosis; immunohistochemical studies have shown a decreased expression of bcl-2 in thyroid follicles from Hashimoto's thyroiditis patients compared to normal controls and also in patients with Graves' disease. Furthermore, interfollicular lymphocytes exhibit weak staining for FasL and strong staining for bcl-2 [128,130,131]. These data suggest that, in patients with Hashimoto's thyroiditis thyroid follicular cells undergo apoptosis by up-regulation of Fas and FasL and down-regulation of bcl-2, which is independent of the antibody-mediated thyroid cell injury.

PATHOLOGY OF HASHIMOTO'S THYROIDITIS The thyroid gland in Hashimoto's thyroiditis is symmetrically enlarged and has a pale pink to yellow, lobulated cut surface (Fig. 4). The accentuation of the lobulations may make the gland appear nodular on gross examination. Characteristic histologic features of Hashimoto's thyroiditis include atrophy of the thyroid follicles with oncocytic (Hürthle cell) metaplasia of the follicular epithelium and abundant lymphoplasmacytic infiltrate with lymphoid follicles including germinal centers. In addition there may be varying degrees of fibrosis and foci of squamous metaplasia associated within the atrophic follicles (Fig. 5). The perithyroidal lymph nodes are generally enlarged and show evidence of reactive lymphoid hyperplasia. The degree of oncocytic metaplasia may vary from focal involvement to diffuse replacement of the follicular epithelium. In some cases there may be aggregates of oncocytic cells forming partially encapsulated hyperplastic nodules [30]. The oncocytic cells may show nuclear enlargement and cytologic atypia. The nuclei of the follicular cells that are associated with the lymphocytic infiltrate may show clearing of the nuclear chromatin and grooves, which may be mistaken for papillary carcinoma [5,30,132,133]. Therefore, strict histologic criteria should be followed in making the diagnosis of papillary carcinoma in the background of Hashimoto's thyroiditis [30].

On immunohistochemistry the lymphocytic population is composed of a mixture of B and T cells. The T cells are predominantly activated CD4+ helper cells with evidence of HLA-DRII expression [99,112]. In addition there are associated CD8+ cytotoxic/suppressor T cells intermixed with T helper cells, B cells, and plasma cells [99]. As mentioned earlier, the CD8+ cells are thought to play a role in Fas–FasL-mediated thyroid follicular cell apoptosis and cytotoxicity. The plasma cells show reactivity with immunoglobulin G (IgG), IgA, and IgM heavy chains and both κ and λ light chains. Immunohistochemistry may also be useful in sometimes differentiating a dense lymphoid infiltrate of Hashimoto's thyroiditis from lymphoma, which may arise in the background of Hashimoto's thyroiditis. The lymphoid infiltrate in Hashimoto's thyroiditis is

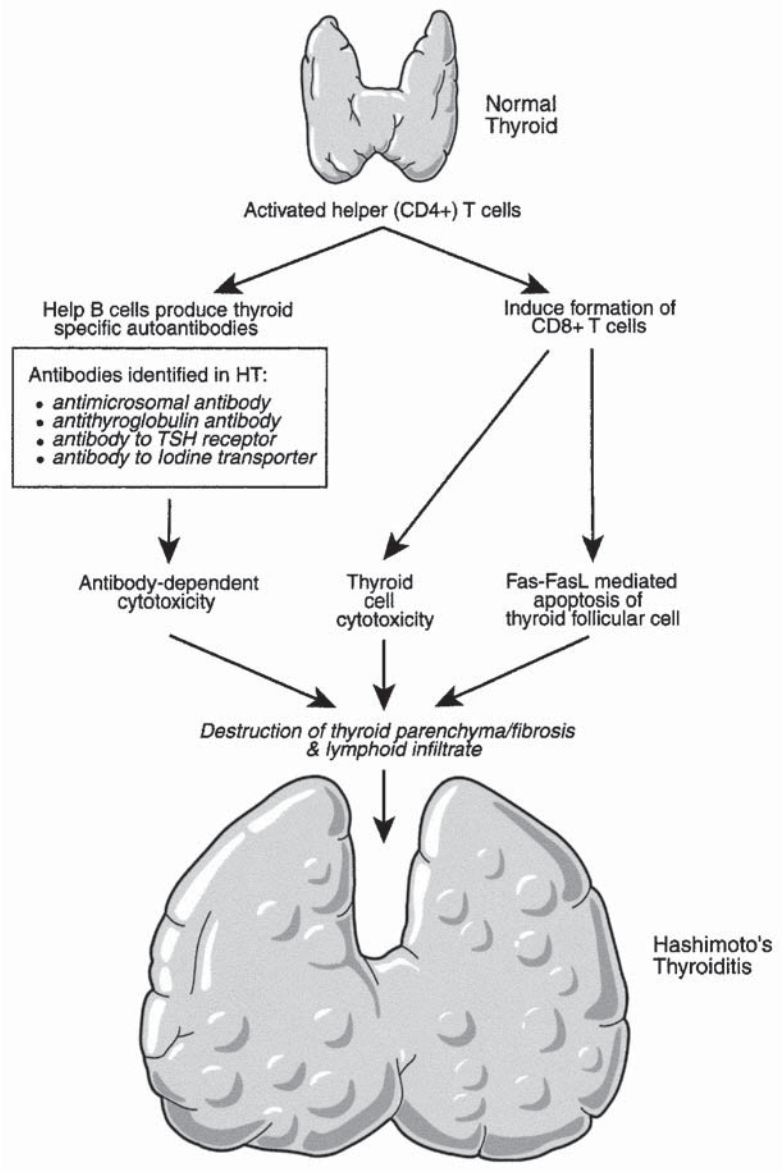

Figure 4 Pathogenesis of Hashimoto's thyroiditis.

polymorphous as described in the preceding and lacks evidence of light chain restriction on κ and λ immunohistochemistry. Additional molecular studies and flow cytometry may sometimes be useful in difficult cases.

PATHOLOGIC VARIANTS OF HASHIMOTO'S THYROIDITIS In addition to the classic form of Hashimoto's thyroiditis described above many variants have been described, which include the following.

Fibrous Variant This comprises approx 10 cases and is seen usually in elderly patients who present with markedly enlarged goiter and hypothyroidism [134,135]. On gross examination the gland is firm in consistency due to marked degree of fibrosis; on microscopy there is extensive destruction of the thyroid follicle with diffuse fibrosis (Fig. 5B), which in some areas has a keloidlike appearance. This fibrotic process is confined to the thyroid capsule, which is an important feature that helps distinguish this form of Hashimoto's thyroiditis from Riedel's thyroiditis. In Riedel's thyroiditis there is involvement of the neck structures such as muscle, nerves, and sometimes the parathyroid by the fibrotic process. Other features that help in this differential diagnosis include lack of vasculitis and myointimal proliferation, which may be seen in Riedel's thyroiditis. Squamous metaplasia may be seen within the follicles. Foci of the classic form of Hashimoto's thyroiditis with lymphoplasmacytic infiltrate and oncocytic metaplasia may also be seen.

Fibrous Atrophy Variant This is also referred to as idiopathic myxedema and is characterized by a very small fibrotic thyroid gland often weighing 2–5 g and is barely identifiable as thyroid on gross examination [5]. There is widespread destruction of the thyroid parenchyma with replacement by fibrous stroma. The histological features are similar to the fibrous variant of Hashimoto's thyroiditis, the only difference being a much smaller gland [4].

Juvenile Variant This is a form chronic lymphocytic thyroiditis seen in younger patients and is often associated with hyperthyroidism, which may later progress to hypothyroidism.

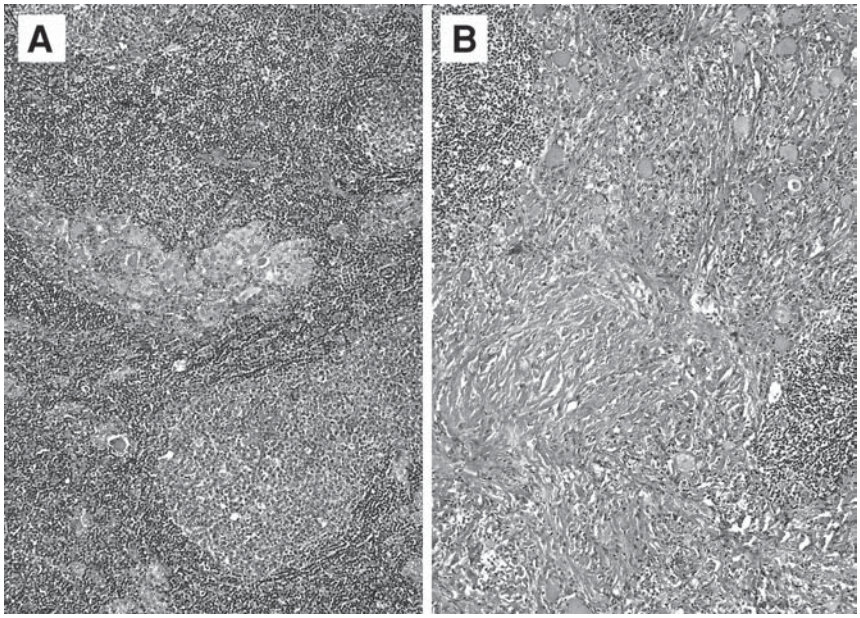

Figure 5 Hashimoto's thyroiditis. (**A**) Classical form with oncocytic metaplasia and marked lymphoid infiltrate. (**B**) Fibrosing variant showing marked fibrosis within the thyroid.

The follicular atrophy and oncocytic metaplasia is focal and hyperplastic changes may be in the thyroid follicles [5].

Subacute Thyroiditis

CLINICAL FEATURES AND ETIOLOGY Subacute thyroiditis, also referred to as granulomatous thyroiditis or de Quervain's thyroiditis, was first described by Mygind in 1895 as "thyroiditis akuta simplex" [136]. The etiology of this form of thyroiditis is unknown, but there is some indirect evidence of association with viral infections. Subacute thyroiditis often follows an upper respiratory tract infection and may include a prodromal phase of muscular aches and pains and fever. It has been associated with infections by adenovirus, Coxsackievirus, Epstein–Barr virus, and influenza virus [136,137]. It has also been seen to be associated with immunosuppressive therapy, suggesting an immune-mediated etiology [138]. Typically patients present with thyroid pain, tenderness, high fever, and ESR. Subacute thyroiditis is a self-limiting disorder in which clinically three phases of the disease are recognized including the hyperthyroid phase, hypothyroid phase, and recovery phase [139,140]. In the hyperthyroid phase, which is the first one to occur, there is an increase in levels of T4 and T3 associated with low radioactive iodine uptake. This thyroid function is a result of excessive destruction of the thyroid follicles, causing increased release of the hormone leading to hyperthyroidism. This progresses to the hypothyroid phase after a significant proportion of the gland has been destroyed. The final phase is that of recovery in which a euthyroid state is established after several weeks or months. Permanent hypothyroidism is very unusual in subacute thyroiditis [84].

PATHOLOGY OF SUBACUTE THYROIDITIS The thyroid gland in subacute thyroiditis is asymmetrically enlarged and is firm in consistency. The cut surface shows a firm tan gland with variable-sized nodules [4]. On microscopic examination in the early hyperthyroid phase there is disruption of the follicles with depletion of the colloid associated with a few multinucleated giant cells. There may be acute inflammation and microabscess formation. In the hypothyroid phase the follicular epithelium disappears and there is a mixed inflammatory infiltrate composed of lymphocytes, plasma cells, and histiocytes including multinucleated giant cells forming granuloma centered around ruptured follicle; colloid may be seen within the giant cells. During the recovery phase there is regeneration of the follicles and fibrosis [4].

Riedel's Thyroiditis

CLINICAL FEATURES AND ETIOLOGY This is a rare disorder causing hypothyroidism due to the destruction of the thyroid gland and its replacement by fibrous tissue. This extensive fibrotic process extends into the extrathyroidal tissues of the neck and therefore this entity has sometimes been correctly referred to as *invasive fibrous thyroiditis* [141]. The clinical presenting features include a rapidly enlarging hard neck mass causing compression of the trachea and esophagus mimicking carcinoma [142].

Hypocalcemia due to hypothyroidism may sometimes be present due to the fibrous destruction of parathyroid [143]. The etiology of Riedel's thyroiditis is not known; autoimmune inflammation has been suggested because of the lymphoplasmacytic infiltrate in the thyroid and the presence of thyroid autoantibodies that may be seen in patients with Riedel's thyroiditis [143]. In view of the extensive fibrosis, Riedel's thyroiditis is considered to be part of the idiopathic fibrosing disorders, which include retroperitoneal and mediastinal fibrosis. These latter disorders have been found to be associated with or may develop after the diagnosis of Riedel's thyroiditis [84].

PATHOLOGY OF RIEDEL'S THYROIDITIS The thyroid gland in Riedel's thyroiditis is enlarged and hard in consistency due to extensive asymmetrical fibrosis. This has been often referred to as "woody thyroid." The fibrosis often extends into the extrathyroidal soft tissues. On the cut surface, the gland is tan-

gray in color with loss of normal lobulations [4]. Microscopy reveals replacement of the normal thyroid parenchyma by dense sclerotic and acellular fibrous tissue. In addition, a mixed inflammatory infiltrate comprising mainly lymphocytes and plasma cells with few neutrophils and eosinophils may be seen. Vascular changes in the form of intimal proliferation and thrombosis may also be seen. In longstanding cases the surgical resection specimen may sometimes contain only dense sclerotic fibrous tissue with no residual thyroid follicles, making a diagnosis of Riedel's thyroiditis on the basis of histology alone difficult. In these cases a clinicopathological correlation is essential to make the diagnosis of Riedel's thyroiditis.

The differential diagnosis of Riedel's thyroiditis includes a fibrosing variant of Hashimoto's thyroiditis (*see* above) and anaplastic carcinoma. Although this distinction can be more easily made on thyroid resection specimens, small biopsies may sometimes pose a problem. Riedel's thyroiditis is distinguished from a paucicellular variant of anaplastic carcinoma by the absence of nuclear pleomorphism, atypia, and mitoses which are usually seen in the latter [4].

Other Forms of Thyroiditis

POSTPARTUM THYROIDITIS Postpartum thyroiditis is a rare autoimmune disorder characterized by transient hyperthyroidism followed by persistent hypothyroidism associated with lymphocytic infiltrate in the thyroid occurring within the first year after delivery [144,145]. It has been regarded as a variant of Hashimoto's thyroiditis [146]. The autoimmune etiology of postpartum thyroiditis has been supported by the finding of a more common prevalence of the disease among women with HLA-DR3, DR4, or DR5 phenotypes, which is similar to that seen in Hashimoto's thyroiditis. In addition, postpartum thyroiditis is seen associated with thyroid autoimmune disorders such as Graves' disease and primary autoimmune hypothyroidism [145]. Postpartum thyroiditis occurs in women with positive antithyroid peroxidase antibody in early pregnancy, the titers of which decline during pregnancy and then rapidly rise again after delivery [145]. The thyroid gland may be slightly enlarged in a diffuse fashion, and on histology shows lymphocytic infiltrate associated with some follicular disruption and focal hyperplasia [4].

SILENT THYROIDITIS This form of thyroiditis has also been referred to as *atypical subacute thyroiditis, painless thyroiditis, chronic lymphocytic thyroiditis,* and *transient hyperthyroidism with lymphocytic thyroiditis* [4]. It shares common thyroid function abnormality with subacute (de Quervain's) thyroiditis including transiently elevated blood levels of T4 and T3 associated with low radioactive iodine uptake. However, in contrast to the pain and tenderness seen in de Quervain's thyroiditis, patients with silent thyroiditis present with a painless thyroid enlargement [137]. The etiology of silent thyroiditis is uncertain, both autoimmune and viral etiology have been proposed [147]. On gross examination there is slight diffuse enlargement of the thyroid gland. Microscopy shows preservation of the lobular architecture with a varying degree of lymphocytic infiltrate and associated follicular destruction. Oncocytic change may be seen but is uncommon.

FOCAL LYMPHOCYTIC THYROIDITIS This is mostly an incidental finding discovered in either surgically removed thyroid or at autopsy and is characterized by a focal lymphocytic infiltrate with preservation of normal lobular architecture and no significant alteration of thyroid functions. It is seen in 5–20% of adult autopsies, mostly in elderly women [4]. On gross examination no specific changes are seen Microscopy reveals preservation of the follicular architecture with foci of lymphocytic infiltrate which may be associated with germinal center formation in the interfollicular region. Focal lymphocytic thyroiditis may be seen associated with multinodular goiter and thyroid tumors [4].

PALPATION THYROIDITIS Palpation thyroiditis is an incidental finding encountered in surgically resected thyroid specimens. It is characterized by focal disruption of thyroid follicles and breakdown of colloid associated with foreign-body-type giant cell reaction including histiocytes and giant cells. This is believed to be the result of palpation of the thyroid during clinical examination causing traumatic release of colloid from the follicles [148]. Granulomas in the thyroid may be seen in other conditions listed in Table 3.

Goiter The term goiter refers to enlargement of the thyroid gland. The limit between a normal thyroid and goiter is well reviewed by Langer [149]. The generally accepted normal range for thyroid weight is 20–25 g, and a gland more than 30 g is considered enlarged. Goiter may be due to a number of causes such as hereditary deficiency of thyroxin synthesizing enzymes (dyshormonogenetic goiter), inflammatory disorders such as Hashimoto's thyroiditis and Graves' disease; thyroid tumors; and goiters due to iodine deficiency and other goiterogenic agents. In this section the latter group of nonhereditary, noninflammatory, and non-neoplastic lesions is discussed. In this group the goiter may be simple (nontoxic) or toxic, and although toxic goiters are usually nodular, the simple goiter may be diffuse or multinodular.

Simple (Nontoxic) Diffuse and Multinodular Goiter Simple (nontoxic) goiter is defined as diffuse or nodular enlargement of the thyroid gland, which is not associated with thyrotoxicosis and does not result from autoimmune or other inflammatory etiology. Simple goiter is the most common thyroid disorder seen in clinical practice and accounts for most cases of nodular thyroid enlargement. Simple goiter may be endemic or sporadic. The most common cause of simple goiter worldwide is iodine deficiency. In clinical practice a goiter is considered to be endemic if >10% of children ages 6–12 within a population have goiter [1,150]. In both endemic and sporadic goiter multiple factors including relative iodine deficiency, naturally occurring goiterogens and minimal levels of thyroid hormone synthesis enzymes may play a role in goiterogenesis. In many cases of sporadic goiter no definitive cause may be identified.

ENDEMIC GOITER The prevalence of endemic goiter is higher in girls than in boys, increases with age during childhood, and peaks at puberty and childbearing age. The prevalence declines in adults and this decline is greater in men than in women [151]. Iodine deficiency in the diet is the most important factor in the development of endemic goiter, which is supported by the finding of decreased prevalence of goiter following iodine supplementation in the diet. However, a significant prevalence of goiter persists after iodine prophylaxis, suggesting that there may additional naturally occurring goiterogens that may play a role in the causation of endemic goiter. These natural goiterogens include vegetables of the genus *Brassica* which contain

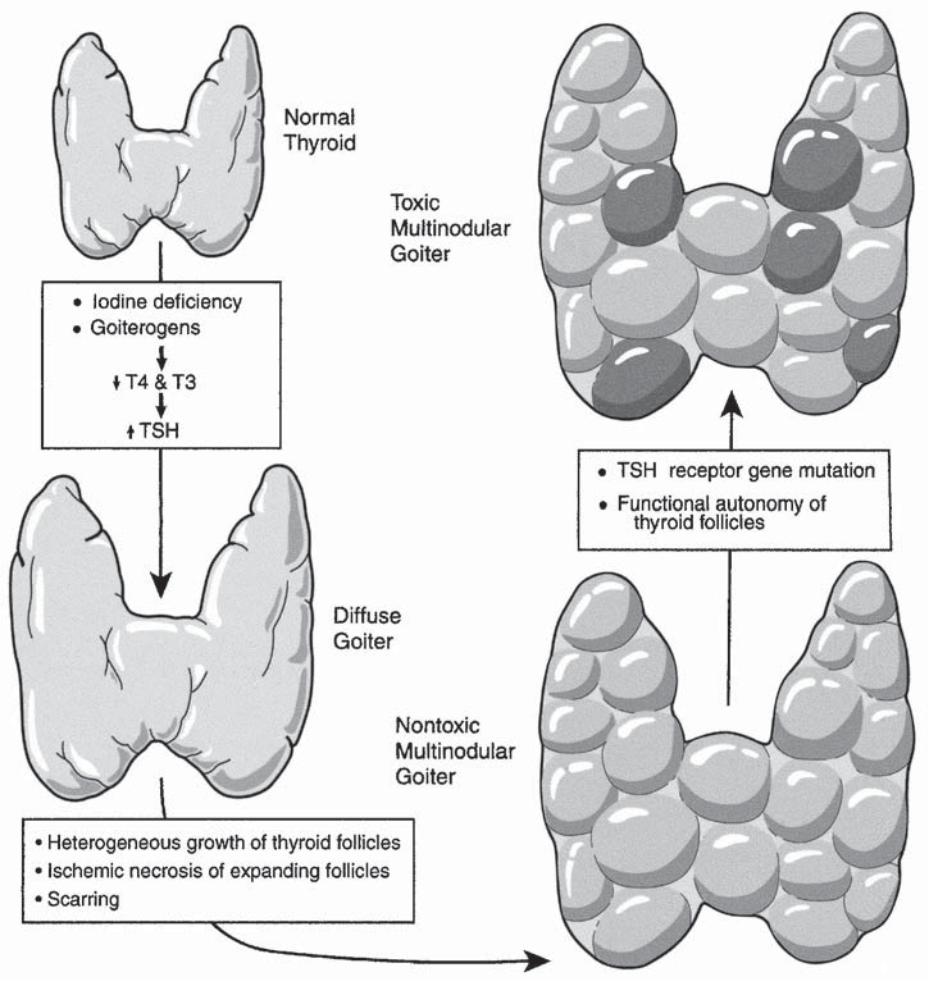

Figure 6 Steps involved in goitrogenesis.

thioglucosides. The thioglucosides on digestion are converted to thiocyanate and isothiocyanate, which is responsible for the antithyroid action. Other natural goiterogens include cassava, maize, bamboo shoots, sweet potatoes, and lima beans, which contain cyanoglucosides that have antithyroid action. The dietary deficiency of iodine and the consumption of natural goiterogens both play a role in the development of endemic goiter [151].

SPORADIC GOITER This type of simple goiter is 10 times more common in women than in men and it occurs around puberty in both sexes and during pregnancy and lactation in women, suggesting its relationship to physiological demand for iodine [1]. The incidence decreases with age in both sexes and there is significant geographic variation in nonendemic goiter areas for the development of sporadic simple goiter [152]. The cause of sporadic goiter is thought to be relative iodine deficiency. In addition, other factors that play a significant role include the presence of dietary goiterogens mentioned above; certain chemicals that interfere with thyroid hormone synthesis, goiterogens; and certain drugs such as paraminosalicylic acid, sulfonylureas, lithium, and excessive iodine [1]. In a large number of patients no cause can be demonstrated [150].

PATHOGENESIS OF SIMPLE GOITER All nontoxic goiters in the early phase of goiterogenesis are diffuse; with time they increase in size and also become nodular. This may be followed by auton-omy in thyroid function independent of TSH secretion causing subclinical thyrotoxicosis and eventually overt thyrotoxicosis [150].

The various stages of goiterogenesis are shown in Fig. 6. In summary these include (1) growth of the thyroid as a result of the stimulatory effect of TSH and growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF), and insulin-like growth factor (IGF); (2) nodule formation due to heterogeneous growth of individual thyroid follicles, and ischemic necrosis of expanding thyroid nodules with associated granulation tissue scarring and calcification; (3) increasing functional heterogeneity within the thyroid in which the capacity of thyroid hormone synthesis varies from follicle to follicle; and (4) the development of functional autonomy in which the follicles within the nodular goiter develop the capacity to synthesize thyroid hormone independent of TSH, leading first to subclinical thyrotoxicosis and finally to overt thyrotoxicosis and toxic multinodular goiter [150].

PATHOLOGY OF NONTOXIC GOITER The thyroid gland in endemic and sporadic goiter shows similar changes. The gland is enlarged in both conditions, but it is larger in endemic goiter compared to sporadic goiter. In early stages the gland is dif-

fusely enlarged, weighing up to 40 g and later in the multinodular goiter stage the weight may range from 60 to 1000 g [4]. The cut surface in the diffuse goiter is homogeneous with a glistening appearance and amber color. In multinodular goiter, the gland is distorted with a nodular surface. On sectioning, the cut surface shows nodules of varying size and a glistening surface. The nodules may be separated by varying degrees of fibrous tissue and have associated areas of hemorrhage and necrosis. Focal calcification and ossification may sometimes be seen. Some nodules have a thick fibrous capsule and need to be differentiated from follicular neoplasms. In some cases a nodule on the surface may become separated from the rest of the gland or be connected by thin fibrous stalk; these are referred to as "parasitic nodules" and should not be misdiagnosed as lymph nodes harboring metastatic carcinoma. Microscopic examination in the earlier hyperplastic stage shows small follicles with very little colloid lined by tall columnar cells, but this stage is rarely encountered in surgical specimens. In the later stage this hyperplasia is followed by involution of the follicles leading to large follicles of varying size filled with abundant colloid; the lining epithelium is flat or low cuboidal. In some large follicles intrafollicular pseudopapillary projections with small lumina may be seen that are referred to as "Sanderstorm's pollsters" [132]. Secondary degenerative changes in multinodular goiter that may be encountered include hemorrhage and necrosis with cholesterol clefts, fibrosis, and foci of calcification and ossification. A dominant nodule with a thick fibrous capsule may sometimes be seen that may have follicles smaller in size than the rest of the gland. These have been referred to as adenomatoid nodules, hyperplastic nodules, or adenomatoid goiter. The differential diagnosis of such a nodule includes follicular adenoma. The features that favor hyperplastic nodule over follicular adenoma include multiple nodules, varying degrees of encapsulation as opposed to thick fibrous capsule, and variable morphology of the follicles within the nodule as opposed to a uniform histology seen in adenoma. Additional molecular diagnostic studies have been used to distinguish hyperplastic nodules from follicular adenoma. These studies have shown that although the majority of hyperplastic nodules are polyclonal, both monoclonal and polyclonal populations may coexist within multinodular goiter [153–157]. One study by Chung et al. [158] suggested that the degree of encapsulation of solitary nodules in multinodular goiter is important for predicting monoclonality; therefore, solitary nodules with a thick fibrous capsule are more likely to be monoclonal. As mentioned earlier, during goiterogenesis from the diffuse to the nodular stage, heterogeneity in the growth of follicles is considered to be the basis of nodule formation, and during this process expansion of a particular clone of thyroid follicular cells with growth stimulatory properties may proliferate leading to nodule formation [150]. It is possible that a hyperplastic nodule may be the preneoplastic stage during thyroid tumorigenesis in which neoplastic transformation of a particular clone of thyrocytes occurs leading to the formation thyroid tumor.

THYROID TUMORS IN NODULAR GOITER Thyroid tumors, both benign and malignant, can be seen in colloid goiter with both solitary and multiple nodules. The prevalence of thyroid carcinoma reported in various studies ranges from 5% to 15% in multinodular goiter and from 8% to 17% in solitary colloid nodules [159–163]. The incidence of carcinoma in multinodular nodular goiter is higher in males compared to females and usually occurs in older age groups [159–161]. The most common malignant tumor arising in multinodular goiter is papillary carcinoma; however, other tumors such as follicular carcinoma, Hürthle cell carcinoma, and medullary carcinoma are also sometimes encountered [162,164]. In a large prospective cohort study of more than 5500 cases of thyroid carcinoma treated in the United States in 1996, a personal history of goiter was the strongest risk factor for the development of carcinoma, which was 14.3% for papillary carcinoma and 15.9% for follicular carcinoma [165]. Multiple foci of follicular pattern lesions with nuclear features of papillary carcinoma may be seen in the background of colloid nodules [166]. In longstanding nodular goiter, the incidence of carcinoma is higher in enlarging solid nodules compared to palpable nodules that decreased in size [167]. Therefore, multinodular goiter with enlarging solid nodules in elderly males carries the highest risk of malignancy, should be closely followed with fine needle aspiration biopsy (FNAB). Lobectomy may be performed if the FNAB is inconclusive. An intraoperative pathology consultation with frozen section and cytology may be helpful if the FNAB is suggestive of papillary carcinoma to guide in further intraoperative management [168].

Toxic Multinodular Goiter Longstanding nontoxic multinodular goiter may become autonomous and produce high levels of thyroid hormone independent of TSH leading to toxic multinodular goiter. The term toxic multinodular goiter includes a spectrum of clinical entities such as a solitary hyperfunctioning nodule within an enlarged nodular thyroid having additional nonfunctioning nodules, to multiple hyperfunctioning areas scattered throughout the gland, barely distinguishable from nonfunctioning nodules [169]. In some cases toxic adenoma that are also autonomous and clonal may be seen in the background of multinodular goiter. Mutations in the thyrotropin receptor gene leading to constitutive activation of the TSH receptor have been found in both toxic adenoma and toxic multinodular goiter [169–173]. In addition constitutively activating mutations in the G_S α-subunit have been seen in toxic adenoma [173,174].

Pathological changes in toxic multinodular goiter and nontoxic multinodular goiter show some similarity on both gross and microscopic examination and it may be difficult on histology alone to make that distinction. Therefore, correlation with clinical features and imaging findings is essential to make that distinction. However, the toxic nodules within multinodular goiter are more cellular, composed of follicles with decreased amounts of colloid and tall columnar cells lining the follicles. These hyperplastic nodules are present in the background of cold nodules similar to the situation in nontoxic goiter.

Other Non-Neoplastic Thyroid Disorders

Effect of Drugs on the Thyroid

AMIADARONE Amiadarone is an iodine-rich drug comprising 37% iodine by weight that is widely used in the management of cardiac arrhythmias and congestive heart failure. Owing to its rich iodine content, thyroid dysfunction is one of the important side effects of amiadarone therapy. The effects of amiadarone on the thyroid have been reviewed by Bogazzi

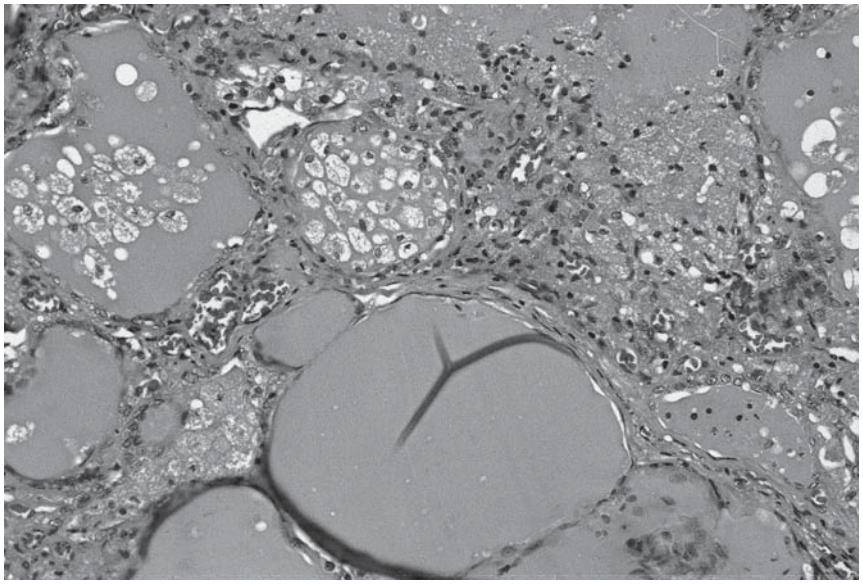

Figure 7 Thyroid gland in amiadarone-associated thyrotoxicosis. Large and degenerating follicles containing foamy cells and evidence of foamy histiocytes and fibrosis in the stroma.

et al. [175]. These may include a euthyroid state to hypothyroidism and overt thyrotoxicosis. Amiadarone-induced thyrotoxicosis (AIT) is due to the release of thyroxin as a result of thyroid follicle destruction. The pathology of AIT is characterized by a diffusely enlarged gland, which on histology shows large involuting follicles with areas of degeneration and destruction of the follicles which are filled with swollen and foamy cells that may contain fine pigmented material. These degenerated follicles are associated with areas of fibrosis and nonspecific chronic inflammation (Fig. 7) [176].

LITHIUM Lithium, which is used in the treatment of bipolar depression, causes subclinical or overt hypothyroidism. Lithium is concentrated in the thyroid and its primary action is to block the release of T4 and T3; it may also inhibit T4 and T3 synthesis [177].

Pigment in the Thyroid The following pigments may be encountered in the thyroid.

IRON Iron is commonly seen in the thyroid in areas of hemorrhage in a nodular goiter or around a previous biopsy site. In these situations iron is seen as a coarse brown hemosiderin pigment deposited in macrophages or the stroma [5]. In some metabolic disorders related to iron such as transfusion-associated hemosiderosis or primary hemochromatosis, excessive iron pigment is seen deposited in thyroid follicular cells [5], which may on rare occasion cause the gland to appear dark brown on gross examination

MINOCYCLINE-ASSOCIATED PIGMENT The antibiotic minocycline is a tetracycline derivative. Prolonged treatment with this drug causes a striking black discoloration of the thyroid referred to as " the black thyroid." Histology shows a granular black pigment within the apical portion of the thyroid follicular cells [5,178]. However, tumors arising in the "black thyroid" lack the black pigment that is seen only in the residual thyroid [179].

LIPOFUSCHIN As in other organs lipofuscin may be seen in the thyroid follicular cells as yellow to light brown cytoplasmic granular material. The clinical significance of lipofuscin accumulation in the thyroid is uncertain, and in most cases no thyroid dysfunction is seen.

THYROID TUMORS The majority of thyroid tumors are epithelial in origin and can be derived from either the follicular cells or the C (parafollicular) cells. As in any other organ site, follicular tumors can be either benign or malignant. No benign counterpart of the C-cell tumor is identified; however, various degrees of C-cell hyperplasia represent the precancerous stage of medullary carcinoma. A classification of thyroid tumors is outlined in Table 4. One of the features in the classification of malignant tumors that seems to be unique to the thyroid is that it is based on the grading of follicular tumors. For example, insular and anaplastic carcinomas are poorly differentiated and undifferentiated stages of a well-differentiated follicular or papillary carcinoma. However, as these subsets of thyroid carcinoma are well-recognized entities and have been followed up for a number of years, having established their specific statistical profile based on clinical and pathological features, we feel that they should be retained for the present. It is possible that in the future these may be replaced by a molecular classification of thyroid tumors when their molecular profiles and clinical correlation are well established.

Follicular Adenoma

Clinical Features and Etiology Follicular adenoma is a benign tumor derived from follicular cells. It is characterized by an encapsulated tumor showing a follicular architecture with varying degrees of cellularity. The incidence of follicular adenoma in autopsy series has ranged from 3% to 4% [132,180] and no significant geographic variations exist. Dietary iodine has no significant role in the causation of conventional follicular adenoma. However, one of the rare variants, toxic adenoma, appears to be more common in iodine-deficient areas [181]. Follicular adenoma is more common in females than in males and the patients are usually middle aged. Although adenomas

Table 4
Classification of Thyroid Tumors

Primary Tumors
I. Tumors derived from the follicular cells
  A. Benign
    1. Follicular adenoma
      –Conventional type
        Microfollicular (fetal), normofollicular (simple), macrofollicular (colloid), trabecular/solid (embryonal)
      –Variants
        Hyalinizing trabecular, follicular adenoma with bizarre nuclei, adenolipoma, adenochondroma, aypical adenoma, toxic adenoma, signet-ring cell adenoma
    2. Oncocytic (Hürthle cell) adenoma
  B. Malignant tumors
    1. Papillary carcinoma
      –Conventional type
      –Variants
        Follicular, encapsulated, diffuse sclerosing, solid/trabecular, tall cell, columnar cell, oncocytic, warthin tumorlike, microcarcinoma
    2. Follicular carcinoma
      –Minimally invasive
      –Widely invasive
    3. Oncocytic (Hürthle cell) carcinoma
    4. Poorly differentiated carcinoma
      –Insular carcinoma
      –Other
    5. Anaplastic carcinoma
II. Tumors derived from C cells
    1. Medullary carcinoma (familial and sporadic)
      –Conventional
      –Variants
III. Mixed follicular and C cell tumors (carcinoma)
IV. Nonepithelial tumors
    1. Mesenchymal
    2. Lymphoma
Metastaic carcinoma
Tumorlike lesions

may occur in children and elderly patients, the chances of an alternative diagnosis of carcinoma are higher in this age group [132] and should be ruled out by further sampling and careful histologic evaluation. The most common clinical presentation of follicular adenoma is a painless lump in the thyroid, which may sometimes show a gradual increase in size and become painful because of hemorrhage and necrosis [132]. The patients are usually euthyroid except the rare cases of toxic adenoma that present with hyperthyroidism. On radionucleotide scan follicular adenoma most of the time presents as a "cold" (hypofunctioning) nodule; sometimes it may be "cool" or "warm" (functioning) and very rarely "hot" (hyperfunctioning) in the case of toxic adenoma [132].

Pathology of Follicular Adenoma On gross examination nearly all adenomas are present as a solitary nodule. However, in some instances two or more adenoma may be seen after strict criteria are applied for excluding hyperplastic nodules [132]. One other possibility that should be considered in the case of multiple nodules resembling follicular adenoma is that of an encapsulated follicular variant of papillary carcinoma (*see* below), and careful histological evaluation for nuclear features of papillary carcinoma should be performed. Follicular adenoma are well circumscribed, vary from 1 to 3 cm in size, have a firm and rubbery consistency, and on sectioning show a diffuse homogeneous cut surface varying in color from grayish white to tan [132]. Secondary changes with areas of hemorrhage, degeneration necrosis, calcification, and ossification may be seen. On histologic examination the architectural pattern of follicular adenoma may vary from solid/trabecular to large macrofollicles and accordingly they have been classified as solid/trabecular, microfollicular, normofollicular, or macrofollicular type (Fig. 8A). The solid/trabecular type is also referred to as an embryonal type because of its resemblance to thyroid tissue during the prefollicular embryonal stage of intrauterine life. Similarly the microfollicular type is also called the fetal type. The follicle size in the normofollicular type is the same as that seen in the normal thyroid; therefore, it is also called the simple type and the macrofollicular type is called the colloid type because of the large follicles mimicking hyperplastic colloid nodules. In most instances more than one architectural pattern is seen in follicular adenoma. No clinical significance is attributed to the various subtypes of follicular adenoma. The lining follicular cells are present as a single layer of polygonal cells with distinct cell borders, round to oval uniform nuclei, and eosinophilic to amphophilic cytoplasm. No mitoses are usually seen. Almost all adenomas have a complete fibrous capsule, which may vary in thickness. A very thick capsule should warrant a very careful evaluation of the entire capsule by examining multiple cuts of a section to rule out foci of capsular and vascular invasion. In addition, studies utilizing picrosirius orange-red (PSR) staining techniques for the evaluation of capsular collagen have found qualitative differences in the staining characteristics of capsular collagen in follicular adenoma and follicular carcinoma which may be helpful in the differential diagnosis [182].

Stromal changes in follicular adenoma include areas of edema that are usually seen in the center of the lesion and other changes that may be seen include hemorrhage, degeneration with cholesterol clefts, necrosis, dystrophic calcification, and ossification. In some cases the vascular network within the adenoma may be very prominent and sometimes may show a hemangiopericytomalike pattern. This close intermingling of follicles with blood vessels may sometimes produce artifactual presence of intravascular epithelial cells, which should not be misdiagnosed as vascular invasion [132]. Other histological changes that may be seen in follicular adenoma include foci of squamous metaplasia, which is exceptionally rare [132], areas of spindle cell metaplasia that may range from 1 to >3 cm in size [183], and papillary architecture mimicking papillary carcinoma [184].

Differential Diagnosis of Thyroid Follicular Lesions

MORPHOLOGY Thyroid follicular lesions have been rightly referred to as "the bane of the pathologist" in a recent article by Baloch and LiVolsi [166]. They include capsulated or unencapsulated lesions with a follicular architecture comprising varying sized follicles filled with variable amounts of colloid. The four most important and common lesions that have to be differentiated in this category include *hyperplastic colloid nodule*, *follicular adenoma*, *follicular carcinoma,* and *follicular variant of papillary carcinoma*.

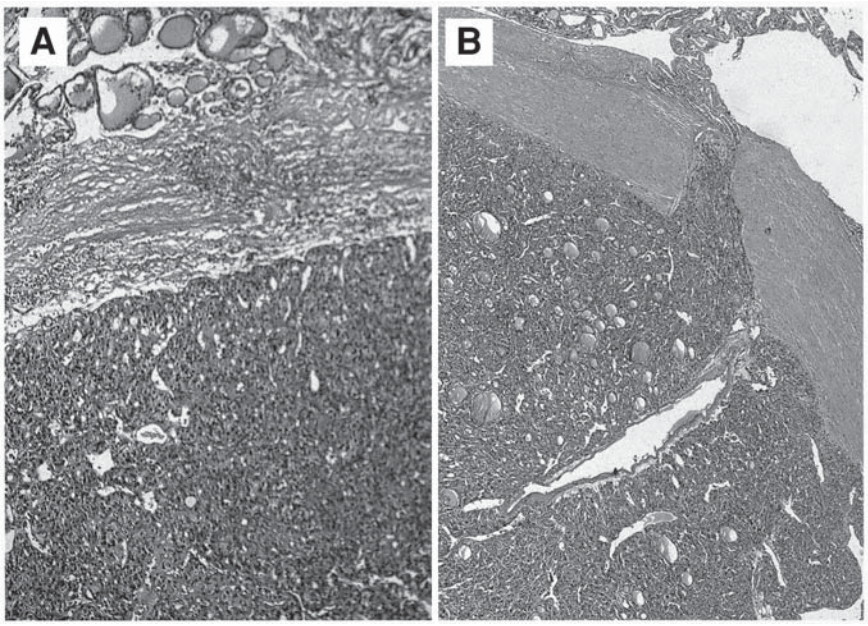

Figure 8 Microfollicular adenoma (**A**) and minimally invasive follicular carcinoma showing a thick fibrous capsule and a focus of transcapsular invasion (**B**).

The features that help in the differential diagnosis of these follicular lesions are outlined in Table 5. Hyperplastic nodules usually lack a well-developed capsule, occur in the background of multinodular goiter, and are mostly macrofollicular or normofollicular. Some lesions may have an incomplete capsule and be microfollicular [166]. While the demonstration of capsular and/or vascular invasion is the sole criterion of distinguishing follicular adenoma from follicular carcinoma, what constitutes capsular and vascular invasion (discussed later) is controversial and lacks consensus among pathologists, surgeons, and endocrinologists [185]. However, a thick fibrous capsule should raise suspicion and warrants a careful and complete evaluation of the tumor capsule in the search for vascular and/or capsular invasion. Multiple cuts of a section may have to be examined. The diagnosis of follicular variant of papillary carcinoma (FVPC) is based on the presence of characteristic nuclear features of papillary carcinoma [132] as discussed later. In addition, the staining quality of the colloid and the presence of stromal fibrous bands may sometimes be helpful in the diagnosis of FVPC [132].

IMMUNOHISTOCHEMISTRY A number of immunohistochemical markers have been studied to evaluate thyroid follicular lesions and to differentiate benign from malignant lesions. One of the markers that have been found to be useful is galectin 3, which is positive in follicular carcinoma and negative in follicular adenoma and hyperplastic nodule [186–193]. In a large multicenter study, the sensitivity and specificity of galectin 3 immunostaining in the diagnosis of thyroid carcinoma was 99% and 98%, respectively. In view of these data, Bartolazzi et al. [191] suggested that if an encapsulated follicular tumor (adenoma) was galectin 3 positive but did not show evidence of capsular and/or vascular invasion on morphology, it is possible that malignant transformation in these cases may have taken place at the molecular level preceding the morphological alteration associated with malignancy. They referred to these tumors as suspicious adenomas or potential early thyroid cancers with molecular evidence of transformation (PETC-MET). Additional immunohistochemical studies that have been found useful in this differential diagnosis include HBME-1, which is strongly positive in follicular carcinoma and negative or focally positive in hyperplastic colloid nodule and follicular adenoma [194–196]. Cell cycle regulatory proteins such as p27 are downregulated in follicular carcinoma and the follicular variant of papillary carcinoma and are diffusely positive in hyperplastic nodules and follicular adenoma [197,198]. Cytokeratin 19 and CD57 are also positive in the follicular variant of papillary carcinoma and negative or weakly positive in hyperplastic nodules and follicular adenoma [196,199–202]. However, in a recent study [203] a subset of follicular adenomas were also positive for CK 19 along with follicular carcinoma, suggesting caution in relying on one single marker for the differential diagnosis of benign vs malignant follicular lesion. Some authors have suggested that a panel including CK-19, HBME-1, and *RET/PTC* [196], *CK-19, HBME-1,* and *CD57* [202] improves sensitivity and specificity in differentiating benign and malignant follicular lesions of the thyroid. In our laboratory we use a panel of *CK-19, HBME-1*, and *galectin-3* for the same purpose in difficult lesions. In view of the recent data [204,205] *PAX-8-PPARγ* may also be added to such a panel of immunostains, as this oncogene has been found to be expressed in follicular carcinoma and not in follicular adenoma. However, one recent study [206] showed diffuse nuclear immunoreactivity for *PPARγ* in 5/16 (31%) follicular adenomas and 7/9 (77%) follicular carcinomas. Therefore more studies with a larger number of cases preferably involving multiple centers may be needed to validate some of the earlier data. Retinoblastoma protein expression is decreased in the follicular variant of papillary carcinoma compared to follicular adenoma [207]. In problematic cases of encap-

Table 5
Differential Diagnosis of Thyroid Follicular Lesions

	Diagnosis			
Pathologic features	*Hyperplastaic nodule*	*Follicular adenoma*	*Follicular carcinoma*	*Follicular variant of papillary carcinoma*
Macroscopic appearance	Usually multiple nodules of varying size	Usually solitary nodule	Usually solitary nodule	May be solitary or multiple nodules
Microscopic appearance				
Capsule	Usually absent, sometimes may be present	Continuous fibrous capsule of varying thickness	Thick capsule with evidence of invasion	May or may not be present
Vascular invasion	Not present	Not present	May be present	May be present
Architecture	Usually normo-macrofollicular	Usually micro- or normofollicular	Usually micro- or normofollicular	Usually micro- or normofollicular
Colloid	Amphophilic or pale eosinophilic	Amphophilic or pale eosinophilic	Amphophilic or pale eosinophilic	Diffusely eosinophilic
Lining cells	Single layer of flat to low cuboidal	Single layer of uniform polygonal	Polygonal cells with cellular areas	Multilayered polygonal cells
Nuclei	Round uniform normochromatic	Round uniform normochromatic	May show mitoses, pleomorphism, and prominent nucleoli	Nuclear grooves, clear chromatin, pseudoinclusions
Ancillary studies				
Immunohistochemistry				
CK19	Usually negative	Usually negative	Usually negative	Strongly positive
HBME	Focally positive	Focally positive	Strongly positive	Strongly positive
Galectin 3	Usually negative	Usually negative	Usually positive	Usually positive
CD57	Usually negative	Usually negative	May be positive	Usually positive
P27	Strongly positive	Strongly positive	Focally positive	Focally positive
Rb protein	Positive	Positive	Negative	Negative
Clonality	Usually polyclonal, may be monoclonal	Usually monoclonal	Usually monoclonal	Usually monoclonal
Cytogenetics				
RET rearrangement	Absent	Absent	Absent	Present in some cases
PAX8/PPARγ rearrangement	Absent	Absent	Present in some cases	May be present in some cases

sulated follicular tumors, these immunohistochemical markers may not be sufficiently discriminatory because they may sometimes be expressed only focally even in classic papillary carcinoma [208]; therefore, careful morphologic evaluation is still the most important in the differential diagnosis of follicular thyroid lesions.

Follicular Adenoma Variants

Hyalanizing Trabecular Adenoma Hyalanizing trabecular adenoma (HTA) is a benign follicular tumor first described by Carney et al. in 1987 [209]. It is composed histologically of islands of polygonal to fusiform cells arranged in a trabecular pattern surrounded by a thin capillary network separated by hyalanized stroma, which is often calcified (Fig. 9). This pattern is reminiscent of a paraganglioma; hence the term paragangliomalike adenoma of the thyroid (PLAT) has been suggested by some [210]. The cells within these trabecular islands show longitudinal grooves, pseudoinclusions, and psammoma bodies; paranuclear yellow cytoplasmic bodies may also be seen [209,211,212]. In view of these nuclear features, which resemble papillary carcinoma, HTA has been the subject of debate among thyroid pathologists and some have suggested that HTA is a variant of papillary carcinoma. Their morphological observations were supported further by the finding of a similar cytokeratin immunoprofile between papillary carcinoma and HTA, especially cytokeratin-19, which was positive in both [213, 214]. These findings, however, have not been reproduced in other studies. Hirokawa et al. [215] reported a completely different cytokeratin profile in HTA and papillary carcinoma; in their study HTA were all CK-19 negative, while papillary carcinoma were positive. In addition, another recent study used MIB staining to differentiate between HTA and papillary carcinoma. The peculiar pattern of cell membrane and cytoplasmic MIB-1 staining seen in HTA was not observed in papillary carcinoma [216]. *RET/PTC* rearrangement has been reported in HTA, suggesting its similarity to papillary carcinoma at the molecular level [214]. More recently, Lloyd has reviewed the status of HTA with respect to *RET/PTC* rearrangement and emphasized the limitations and practical usefulness of these analyses [217] (discussed further in Chapter 10.) We think that in the absence of any clinical correlation with respect to immunophenotypic and cytogenetic similarities between HTA and papillary carcinoma it is difficult to draw any definitive conclusions. Therefore, we feel that in the absence of capsular and/or vascular invasion this tumor should continue to be classified as a benign follicular tumor and strict morphologic criteria used to distinguish this from a solid/trabecular variant of papillary

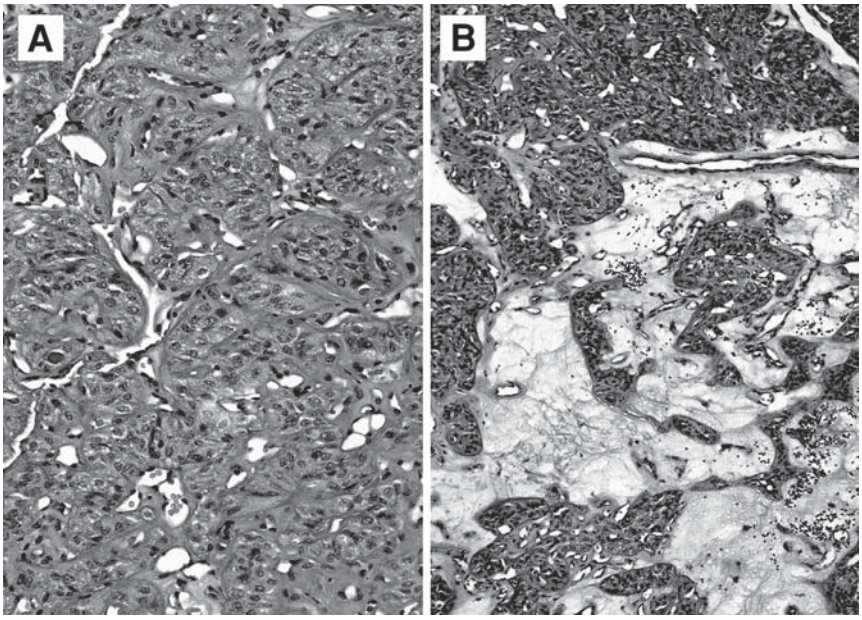

Figure 9 Hyalanizing trabecular adenoma. (**A**) Peripheral portion of the tumor; (**B**) central portion of the tumor showing stromal hyalinization and edema.

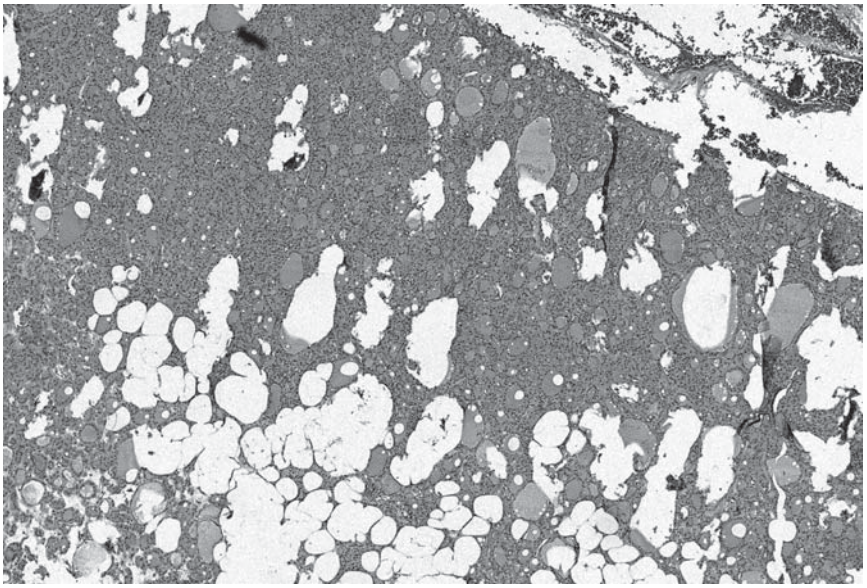

Figure 10 Adenolipoma of the thyroid showing interspersed mature fat within the follicular lesion.

carcinoma. It is possible that these tumors may represent a group of thyroid tumors with very low malignant potential with an uncertain biology [218], and further studies utilizing molecular diagnostic techniques with a clinical correlation may shed more light in the future.

Atypical Follicular Adenoma This term was proposed by Hazard and Kenyon in 1954 for follicular adenoma having some unusual features such as closely packed follicles lacking lumina, solid columns, with little intervening stroma and diffuse cellularity [132]. Pathologists have come to include in this category adenoma showing necrosis and increased mitotic activity [132]. In such cases careful examination of the capsule should be performed to rule out capsular or vascular invasion.

Follicular Adenoma with Bizarre Nuclei In this variant of follicular adenoma scattered large nuclei more than 10 times the size of adjacent cells are seen. These nuclei are hyperchromatic and irregular in shape and do not represent a sign of malignancy on their own [132].

Adenolipoma and Adenochondroma Follicular adenoma with interspersed mature fat in between follicles are termed adenolipoma (Fig. 10) and accordingly tumors with islands of cartilage are called adenochondroma [132,219].

Toxic Adenoma Most conventional adenomas are hypofunctioning or "cold" on radionucleotide scan. Toxic adenoma is a rare variant comprising approx 1% of all follicular adenoma, which is "hot" on radionucleotide scan, and the patients

present with hyperthyroidism. It is also referred to as Plummer's disease [132]. On microscopic examination toxic adenoma is either microfollicular or normofollicular and contains pseudopapillary projections. The cells lining the follicles are tall columnar, and there is decrease in the amount of luminal colloid within the follicles [132]. Ultrastructurally, the cells show features similar to those seen in Graves' disease, which include increase in rough endoplasmic reticulum, well-developed Golgi apparatus, numerous lysosomes, and numerous slender apical microvilli and pseudopods [132]. At the molecular level, there is mutation in the TSH receptor gene causing constitutive overexpression of the TSH receptor leading to hyperfunctioning of the thyrocytes [169–171,173,174].

Signet-Ring Cell Follicular Adenoma This variant of follicular adenoma is characterized by the presence of large intracytoplasmic vacuoles displacing the nuclei to one side causing the signet-ringlike appearance [220–222]. These foci with signet-ringlike areas may be focal or diffuse throughout the lesion. The cytoplasmic vacuoles contain thyroglobulin, and on ultrastructural evaluation these vacuoles represent either intracellular lumina or dilated vesicles [30]. Mucin stain may sometimes be positive within the intracytoplasmic vacuoles, and this is thought to be due to glycoprotein complexes produced as a result of thyroglobulin degradation [222].

Thyroid Carcinoma Thyroid carcinoma, which constitutes >98% of thyroid malignancies [223], accounts for 1% of all human cancers [165]. At a global level there is significant geographical variation in the incidence of thyroid carcinoma, which ranges from 0.5 to 10 cases per 100,000 [30]. In the United States and Europe, the incidence is about 3 per 100,000 population [223]. Females are three times more commonly affected than males [165]. Although thyroid carcinoma can be seen in all age groups, the most common ages at onset are the fourth and the fifth decades. Most thyroid carcinomas are differentiated tumors and have an indolent course. Anaplastic carcinoma, which is one of the most aggressive forms of thyroid carcinoma, is most commonly seen in the elderly, mainly in the seventh and the eighth decades of life [165]. Thyroid carcinoma can arise from either the thyroid-hormone-producing follicular cells or the calcitonin-producing C cells. The latter are referred to as medullary carcinoma and may either be sporadic or arise in a familial setting as part of multiple endocrine neoplasia (MEN). The incidence of different types of thyroid carcinoma may vary worldwide depending on the iodine content of the diet. In a large multicenter prospective cohort study [165] including more than 5500 cases in the United States, among the follicular-cell-derived thyroid carcinoma, the most common is papillary carcinoma (81%), followed by follicular carcinoma (10%), Hürthle cell carcinoma (3.6%), and anaplastic carcinoma (1.7%). The incidence of medullary carcinoma in this group of cases was 3.2% (2.7% sporadic and 0.5% familial). Nonmedullary thyroid carcinoma including papillary, follicular, and anaplastic carcinoma may also occur in a familial setting [224–226]. In iodine-deficient areas the incidence of follicular carcinoma is higher and may be as high as 45% [30]; however, in parts of the world with sufficient iodine supplementation the incidence of follicular carcinoma has declined over the past few decades. One reason in addition to iodine supplementation may be an increase in the incidence of papillary carcinoma, especially its follicular variant [227].

Papillary Thyroid Carcinoma

CLINICAL FEATURES AND ETIOLOGY This is the most common form of thyroid carcinoma with no apparently known benign neoplastic counterpart [228]. It most often presents in the fourth and the fifth decades and is three times more common in women than in men [165]. Most thyroid tumors in children are papillary carcinoma and rare reports of congenital tumors are also present [30]. Papillary carcinoma may sometimes be seen in a familial setting [30,225], and family history of thyroid carcinoma was seen in 4.9% cases of papillary carcinoma in a large cohort of thyroid cancer [165]. In the same cohort, potential risk factors associated with papillary carcinoma included goiter (14.9% cases), thyroiditis (8.1% cases), history of prior exposure to radiation (4.8% cases), and Graves' disease (2.0% cases) [165]. Somatic rearrangements of the *RET* protooncogene, which is located on the long arm of chromosome 10 and encodes a membrane-associated tyrosine kinase receptor, have been seen in papillary thyroid carcinoma and the rearranged form of the *RET* oncogene is designated as *RET/PTC* gene. This *RET/PTC* rearrangement is believed to play a role papillary thyroid carcinogenesis (for review *see* 229,230). In experimental models *RET/PTC* rearrangements have been observed in mice exposed to ionizing radiation, suggesting the role of ionizing radiation in the causation of papillary thyroid carcinoma [231]. The role of ionizing radiation in the causation of thyroid cancer is well recognized and has been reviewed recently by Moysich et al. [232].

PATHOLOGY OF PAPILLARY THYROID CARCINOMA The typical gross appearance of papillary carcinoma is an ill-defined tumor with irregular borders and firm consistency with granular pale white cut surface, sometimes associated with calcification [132]. Another presentation of conventional papillary carcinoma is a cystic tumor with attached papillary growth. Some variants may show a well-circumscribed nodule with a fleshy cut surface that is often encapsulated and may show partial cystic change [132]. The latter form of presentation on gross examination may resemble an adenoma, and careful microscopic evaluation for nuclear changes of papillary carcinoma should be performed in these cases.

On microscopic examination the architecture of papillary carcinoma may be papillary comprising a complex arborizing papillary process with well-defined fibrovascular cores; it may be follicular, solid/trabecular, or mixed. The hallmarks of the diagnosis of papillary thyroid carcinoma are the characteristic nuclear changes, which are seen in the conventional papillary carcinoma and all its variants (Fig. 11). These changes include elongation of the nuclei with nuclear grooves present parallel to the long axis of the nuclei; clearing of the nuclear chromatin referred to as "orphan Annie" nuclei, and the presence of nuclear pseudoinclusions, which are eosinophilic and represent extension of the cytoplasmic contents that appear to lie in the nucleus due to the highly irregular and undulated nuclear membrane [30,132]. Although nuclear grooves may be seen in other types of thyroid lesions, both neoplastic and non-neoplastic [233], the overall appearance of the lesion both at the architectural and the cytologic level is helpful in distinguishing

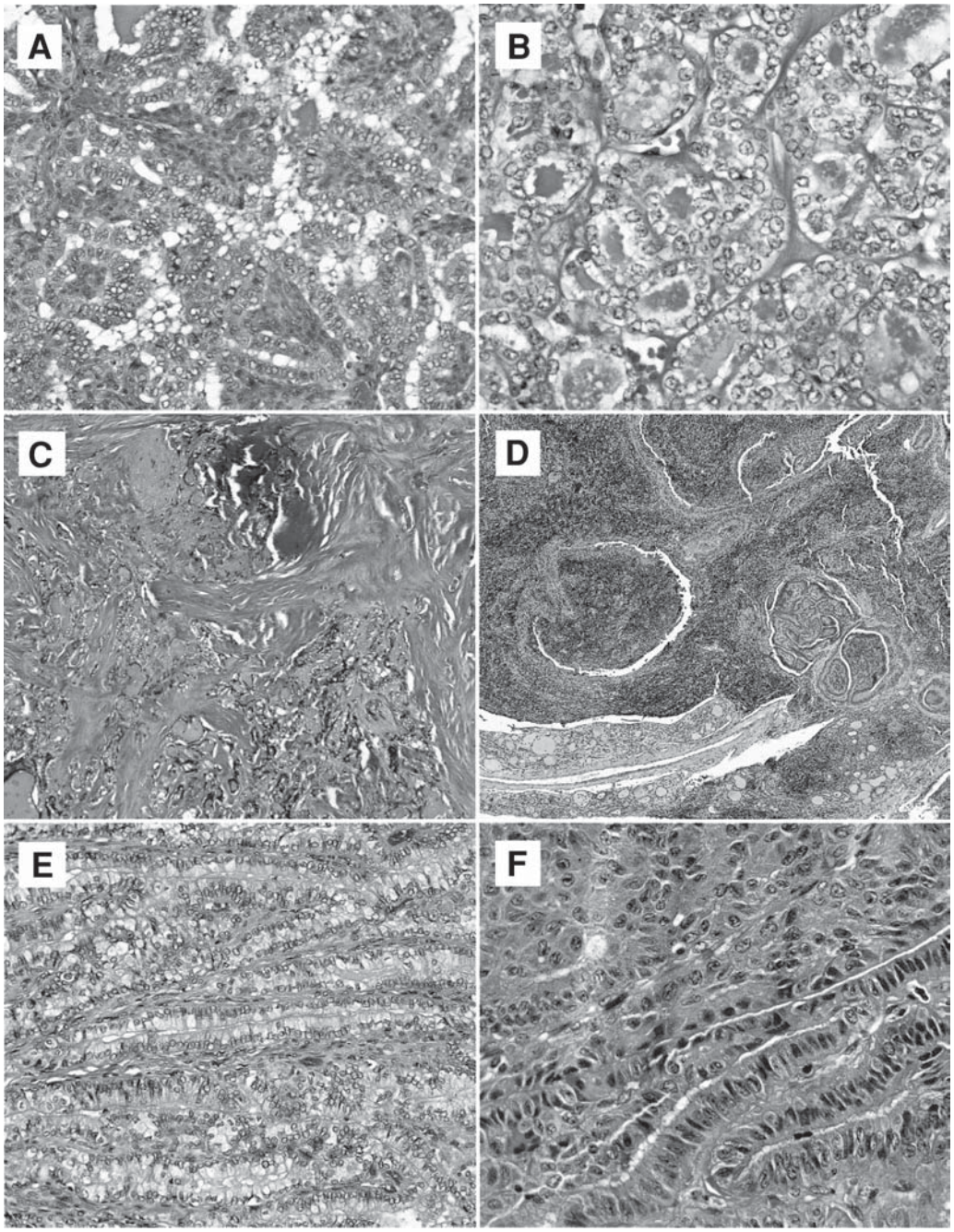

Figure 11 Papillary thyroid carcinoma. (**A**) Conventional type; (**B**) follicular variant; (**C**) diffuse sclerosing variant; (**D**) Warthinlike papillary carcinoma; (**E**) columnar cell variant; (**F**) tall cell variant. (Color illustration appears in insert following p. 148.)

these conditions from papillary thyroid carcinoma. These nuclei in papillary carcinoma often show overlapping features. Additional histologic changes include the presence of psammoma bodies, which are concentric calcific bodies lying in the stroma; and desmoplastic stroma with infiltrative growth of the tumor and variable degree of lymphocytic infiltrate in the stroma. Foci of squamous differentiation may sometimes be seen [30, 132]. Papillary carcinoma may show multicentricity and may be present in bilateral lobes, justifying the near total thyroidectomy that most surgeons prefer, especially for tumors >1.0 cm in size. It is not completely clear whether the multicentricitry is due to intrathyroidal spread of the tumor via lymphatics, or represents multiple synchronous primaries. While some studies have shown that these multiple foci of tumors are clonal, others have shown that they represent separate clones [30]. Papillary carcinoma usually disseminates through lymphatics, and it is not uncommon to find metastases in regional lymph nodes. Sentinel lymph node biopsy has been suggested by some

authors as an accurate method for the diagnosis of metastatic papillary carcinoma, avoiding the significant morbidity associated with lymph node dissection [234,235].

On electron microscopy, the nuclear membrane of papillary carcinoma is highly irregular showing infolding, the chromatin is scant, and nuclear pseudoinclusions containing cytoplasmic are seen [30].

Numerous immunohistochemical markers such as cytokeratin-19, HBME, CD57, CD44, galectin 3, p27, cyclin D1, and retinoblastoma protein have been reported to help in the diagnosis of papillary thyroid carcinoma, especially in follicular patterned lesions [186–203]. While some have shown promising results especially when used in a panel as discussed earlier, no single marker on its own is absolutely specific and morphology still may be the gold standard for the diagnosis of thyroid carcinoma.

HISTOLOGIC VARIANTS OF PAPILLARY CARCINOMA Although the characteristic nuclear features described above are common to all histologic types of papillary carcinoma, a number of variants have been described based on either size or architectural patterns of the tumor (Fig. 11). The diagnosis of some these variants is important because they behave more aggressively compared to conventional papillary carcinoma, and the knowledge of some other variants is important to prevent misdiagnosis.

Papillary Microcarcinoma Papillary thyroid microcarcinoma is defined as tumor <1.0 cm in size; it is also referred to as occult sclerosing carcinoma and is not an uncommon incidental finding at autopsy and in surgical resection of thyroid for benign conditions [30]. Because of its high prevalence, in up to 35% in autopsy series and in up to 24% of total thyroidectomies for unrelated benign conditions [236], this tumor is regarded as an indolent tumor with a relatively benign course [237,238]. For the same reason some people have suggested that this may be an earlier stage in papillary carcinogenesis [228]. However, up to 11% of microcarcinoma may show lymph node metastases and local recurrence, which is usually seen in multifocal and bilateral tumors [30].

Follicular Variant of Papillary Carcinoma This is the most common variant of papillary carcinoma and is also the one that has generated a lot controversy in its diagnosis. Although Crile and Hazard [239,240] had recognized carcinoma with a follicular pattern as early as 1953, the term follicular variant of papillary carcinoma was first proposed by Lindsay in 1960 [241], but it was still regarded as a type of follicular carcinoma. The WHO in 1974 [242] recognized the entity as follicular variant of papillary carcinoma in its classification of thyroid tumors. Later in 1977 Chen and Rosai [243] described six additional cases elaborating the morphologic features and proposed the use of Lindsay's terminology follicular variant of papillary carcinoma. Since the description by Chen and Rosai, this variant of papillary carcinoma is now more readily recognized and in some series 29% of papillary carcinoma have 70% or more of the tumor showing a follicular architecture [244]. The follicular pattern in this tumor may be microfollicular, normofollicular, macrofollicular, or a mixed pattern (Fig. 11B). These tumors may be partially or completely encapsulated. One of the most controversial and challenging diagnoses in thyroid surgical pathology may be the identification of focal nuclear changes of papillary carcinoma in an encapsulated follicular pattern lesion that many pathologists may have faced [245]. It has been suggested that there may be a tendency to overdiagnose follicular variant of papillary carcinoma [246], and, furthermore, if these encapsulated follicular lesions with apparently equivocal nuclear changes of papillary carcinoma are sent out for consultation, more often than not divergent opinions may be obtained, which reflects the lack of uniform diagnostic criteria [247]. Because the nuclear changes are not diffuse, they may sometimes be missed and the lesion may be misdiagnosed as follicular adenoma. These nuclear changes are most often seen in the subcapsular region of the tumor [30]. The importance of diagnosing these lesions as an encapsulated follicular variant of papillary carcinoma cannot be overemphasized because these tumors may later present with bone metastases, some 15–17 yr after the initial diagnosis, which justifies their appropriate treatment at the time of presentation [248]. In view of this difficulty in their diagnosis, some have suggested that encapsulated follicular lesions with questionable nuclear changes be diagnosed as well-differentiated tumor of uncertain malignant potential (WDT–UMP) [245]. However, as these tumors may later present with bone metastases as mentioned above, treating them as carcinoma at initial presentation may be justified. The features that may help in the differential diagnosis of these follicular pattern lesions are outlined in Table 5.

In addition to the encapsulated pattern of FVPC, other distinct patterns that have been described in FVPC include a diffuse variant that involves the entire thyroid and shows a high incidence of lymph node and distant metastases [30,132,249] and the macrofollicular variant described by Albores-Saavedra et al. that should be distinguished from hyperplastic colloid nodule [250]. Some macrofollicular variants in a later publication by the same authors showed focal transformation into a poorly differentiated (insular) phenotype with evidence of lung and bone metastases [251].

Although some cases as described above have shown evidence of lung and bone metastases, in general the natural history of FVPC is the same as that of conventional papillary carcinoma, especially with respect to spread to the lymph nodes and prognosis. Furthermore, immunophenotypically the keratin expression profile of these tumors resembles that of conventional papillary carcinoma as opposed to the follicular carcinoma [132].

Tall Cell Variant of Papillary Carcinoma This is one of the more aggressive variants of papillary carcinoma, first described by Hawk and Hazard in 1976 [252]. These tumors usually present in the elderly and are more common in males than in females [30]. On gross examination, the are usually >5.0 cm in size and on histologic examination show papillary structures lined by elongated tumor cells, with the height being at least twice the width and having an eosinophilic cytoplasm and nuclear features of papillary carcinoma (Fig. 11F). Ultrastructurally there are increased mitochondria in the cytoplasm, but less than that seen in Hürthle cells [30]. The tall cell variant of papillary carcinoma is associated with poor prognostic features such as large size, extrathyroiodal extension, vascular invasion, and shows a high incidence of recurrence and shorter disease-free survival and distant metastasis [253–257]. In addition, based

on cell cycle regulatory protein such as p27, MIB1 and cyclin D1, p53, and eukaryotic translation initiation factors 4E and 2α expression, they show a profile that is similar to that seen in thyroid tumors with an unfavorable prognosis [258–262]. The tall cell variant have been found to be associated with squamous cell and Hürthle cell carcinoma of thyroid, both of which are more aggressive phenotypes [261,263].

Columnar Cell Variant of Papillary Carcinoma This is a also a more aggressive variant of papillary carcinoma first described by Evans [30]. LiVolsi later highlighted an important morphologic alteration in these cells which included the subnuclear vacuolation (Fig. 11E) similar that seen in early secretory endometrium [5]. This latter feature is important and helps in differentiating this from the tall cell variant. These tumors have been found to be associated with the tall cell variant and have an adverse clinical course [262].

Cribriform Variant of Papillary Carcinoma This is a rare but distinct variant of papillary carcinoma that is seen associated with familial adenomatous polyposis (FAP) and germ-line mutations in the *APC* gene [264–268]. Sporadic cribriform variants of papillary carcinoma have also been reported without associated FAP [269]. Cribriform variant of papillary carcinoma occurs almost exclusively in females and may be solitary or multifocal. They may be encapsulated and histologically are characterized by a cribriform, solid/trabecular, morular (squamoid) growth pattern with intermixed papillary and follicular areas. The diagnosis of papillary carcinoma is based on the finding of characteristic nuclear features [30,269]. Immunohistochemistry shows reactivity with thyroglobulin, epithelial membrane antigen, cytokeratin, vimentin, estrogen, and progesterone receptors and behavior of these tumors is similar to that of the conventional papillary carcinoma [269].

Oncocytic Variant of Papillary Carcinoma This variant of papillary carcinoma displays a papillary architecture in which the papillae are lined by cubocolumnar cells with eosinophilic cytoplasm and nuclear features of papillary carcinoma [30]. This tumor should be differentiated from papillary variant of Hürthle cell carcinoma because of the different biology of the two tumors. In the latter, characteristic nuclear features of papillary carcinoma are not seen and the tumor has very little stroma [30].

Solid Variant of Papillary Carcinoma This variant of papillary carcinoma is most often seen in the pediatric population and has been seen as the most common variant of papillary carcinoma in children following the radiation exposure due to the Chernobyl nuclear disaster [270,271]. These tumors are associated with adverse prognostic factors such as lymph node metastases, extrathyroidal extension, and venous invasion. On histology these tumors are composed of solid islands of oval cells separated by thin fibrous septae. The cells show nuclear features of papillary carcinoma. Areas of follicular and papillary architecture may also be seen intermixed [272]. They are associated with slightly higher incidence of distant metastases and less favorable prognosis than conventional papillary carcinoma [273], but they must be differentiated from insular carcinoma which may seen associated with differentiated thyroid carcinoma of both papillary and follicular types and have a worse prognosis [30,132].

Warthinlike Tumor of the Thyroid This variant of papillary carcinoma resembles the Warthin tumor of the salivary gland, and because of this feature Apel et al. termed this variant as "Warthinlike thyroid tumor" [274]. It is associated with lymphocytic thyroiditis and is composed of papillae lined by tall eosinophilic cells with nuclear features of papillary carcinoma separated by abundant lymphocyte rich stroma (Fig. 11D). This tumor behaves like conventional papillary carcinoma [275–277].

Papillary Carcinoma with Nodular Fascitislike Stroma This is a rare variant of papillary carcinoma in which there is marked fibroblastic proliferation in the stroma mimicking granulation tissue, which may sometimes mask the tumor cells. The tumor cells show nuclear features of papillary carcinoma [278–281]. In surgical pathology practice it is important to recognize this variant so that in the presence of exuberant fibroblastic proliferation a careful search should be made to look for tumor islands. Furthermore, this variant must be distinguished and not misdiagnosed as a more aggressive anaplastic thyroid carcinoma [278]. In this variant the presence of fibroblastic stroma may pose problems in its diagnosis on FNAB [282].

Diffuse Sclerosing Variant of Papillary Carcinoma This variant of papillary carcinoma is more common in children and adolescents and shows diffuse involvement of the thyroid by a widely invasive tumor associated with dense fibrous stroma (Fig. 11C). Focal squamous metaplasia, numerous psammoma bodies, and lymphocytic infiltrate are additional histologic features [30,132]. Their behavior is similar to that of conventional papillary carcinoma. Owing to the diffuse infiltrative pattern these tumors may not produce a clinically palpable mass, so these patients tend to present at a later stage [132].

PROGNOSIS IN PAPILLARY THYROID CARCINOMA With the exception of some more aggressive histologic variants mentioned above papillary carcinoma has an excellent prognosis. The overall 5-yr survival rate is 90–95% and the 10-yr survival rate is 80–95%. Independent adverse prognostic factors include older age (>45 yr), extrathyroidal spread, and distant metastases [132,223].

Follicular Carcinoma Follicular carcinoma is a malignant tumor derived from the thyroid follicular cells that shows a follicular architecture and *does not* show the characteristic nuclear features associated with papillary carcinoma. The latter feature is very important and may be one of the reasons for the decline in the incidence of follicular carcinoma over the years ever since the follicular variant of papillary carcinoma gained recognition after its description by Chen and Rosai in 1977 [185,227,243]. However, follicular carcinoma is still the second most common malignant tumor after papillary carcinoma, accounting for 10% of all thyroid cancers in the United States in a recent prospective cohort analyses [165]. In other parts of the world, especially in areas with iodine deficiency, the incidence of papillary carcinoma is higher and may be up to 45% of all thyroid cancers [30]. It has been shown that addition of iodine to the diet results in a relative increase in papillary carcinoma and a corresponding decrease in follicular carcinoma [132]. Follicular carcinoma may occur at any age, but most commonly presents in the fifth decade and has the same female predilection of 3:1 as papillary carcinoma [165,283–285]. It typically presents as a solitary thyroid nodule that is usually

"cold" on radionucleotide scan, sometimes bone metastases may be the presenting feature, and unlike papillary carcinoma, follicular carcinoma are not clinically occult [132].

Follicular carcinoma is subdivided into two groups by the WHO in its classification of thyroid tumors: *minimally invasive or encapsulated* and *widely invasive* follicular carcinoma [286]. The distinction into these two groups is important because of their significantly different clinical behavior and treatment [185, 287]. However, what constitutes minimally invasive follicular carcinoma has been the subject of debate and lacks uniform criteria among pathologists, endocrinologists, and surgeons [185,288].

Minimally Invasive (Encapsulated) Follicular Carcinoma Minimally invasive follicular carcinoma (MIFC) is defined as an encapsulated follicular tumor showing foci of full thickness capsular invasion (Fig. 8B) and/or vascular invasion within or outside the capsule [132]. The capsule in most of these tumors is thick, but cases with thin capsule or uneven poorly formed capsule may also be seen [288]. However, what constitutes *capsular invasion* lacks consensus and both partial and full thickness capsule invasion have been cited as criteria for capsular invasion in the past [30]. A survey of endocrine pathologists revealed lack of consensus on the definition of capsular invasion to diagnose MIFC [185]. In view of this lack of uniformity in the diagnostic criteria, some authors have suggested that encapsulated follicular tumors with questionable capsular invasion showing no vascular invasion should be classified as follicular tumor of uncertain malignant potential (FT-UMP) [245]. There are reports of follicular carcinoma with capsular invasion only showing distant metastases, justifying their designation as carcinoma and appropriate management [289,290]. The presence of *vascular invasion* is regarded as a definite sign of malignancy; the vascular invasion, however, should be in a blood vessel within or immediately outside the capsule [132]. In minimally invasive carcinoma, the blood vessels outside the capsule are of small or medium size and lack a continuous muscular layer [287,288]. The tumor cells should be present within the vascular lumen like a fibrin thrombus and show attachment to the endothelial surface at some point to avoid overdiagnosis by artifactual presence of tumor cells in the blood vessel as a result of contamination during handling of the specimen at surgery or gross examination. At times tumor cells are seen pushing into a thin blood vessel from outside, causing protrusion of the tumor cells into the lumen with intact endothelial cells on the surface. This should also not be regarded as vascular invasion if strict criteria are adopted [132]. LiVolsi has suggested an approach that seems practical in which tumors with capsular invasion only are regarded as minimally invasive carcinoma and tumors with vascular invasion designated as angioinvasive grossly encapsulated follicular carcinomas. The angioinvasive tumors have a capacity for hematogenous spread, and 50% of these patients die of tumor at 10 yr; patients with capsular invasion only have a better prognosis [30]. Further larger series with long-term outcome data applying this classification may be useful in validating its clinical significance. Thomson et al., in series of 95 MIFC, which included cases both with capsular and/or vascular invasion, showed excellent survival. All but one patient were alive after a mean follow-up of 16.8 yr. Four patients showed recurrent disease, one of whom died after 15 yr, this latter patient was a female who was 49 yr old at presentation and had a large 7.0 cm tumor [288]. Distant metastases in MIFC are seen more often in tumors showing vascular or complete vascular invasion [291]. In view of this focal nature of capsular and vascular invasion, it is important that encapsulated follicular tumors should be evaluated by thorough sampling including examination of the entire capsule. In our experience we have found doing multiple serial sections of the capsular region, especially in cases with thick fibrous capsule, to be helpful in identifying foci of capsular and vascular invasion. The differential diagnosis of MIFC includes follicular adenoma, follicular variant of papillary carcinoma, and hyperplastic colloid nodule which has been discussed earlier (*see* Table 5). Furthermore, many ancillary tests using immunohistochemistry and molecular diagnosis techniques as mentioned above have been suggested to be helpful in the diagnosis of equivocal follicular lesions (Table 5). However, thorough sampling and careful morphologic evaluation still may be the best diagnostic method at present, and may be supported by additional ancillary studies in difficult cases.

Widely Invasive Follicular Carcinoma The diagnosis of this subtype of follicular carcinoma is fairly straightforward. On gross examination, the tumor has widely invasive edges with foci of necrosis; microscopy reveals a follicular tumor, often with solid or trabecular areas and invasion of the surrounding thyroid parenchyma. The tumor cells may show high mitotic rate and areas of necrosis may be seen. In some cases there may be transformation into a poorly differentiated (insular) or an anaplastic phenotype indicating the progression of differentiated carcinoma to poorly differentiated and anaplastic phenotype [132]. These tumors spread to distant organs through blood vessels and up to 80% may develop metastases. The prognosis in this tumor is worse than in MIFC. The 10-yr survival in these patients is 25–45% as opposed to 70–100% in the MIFC group [30,132].

Oncocytic (Hürthle Cell) Tumors Thyroid follicular cells with oncocytic feature including finely granular abundant eosinophilic cytoplasm were first described by Askanazy in 1898, the cells that were described by Hürthle in the thyroid of a dog are thought to represent the parafollicular C cells [132]. However, the term Hürthle cell for oncocytic thyroid cells that were actually described by Askanazy has been so ingrained in our minds that people continue to use this terminology. The AFIP fascicle on thyroid tumors [132] has proposed to designate these tumors as oncocytic tumors.

Oncocytic tumors is a rare group of thyroid neoplasms, with oncocytic (Hürthle cell) carcinoma comprising 3.6% of all thyroid cancers in the United States [165]. Although derived from thyroid follicular cells, oncocytic carcinoma has a distinct oncogenic expression, which is different from follicular and papillary carcinoma [292]. While these tumors are divided into two categories, the benign tumor as adenoma and the malignant counterpart as oncocytic carcinoma [293], some of the earlier studies suggested that all oncocytic (Hürthle cell) neoplasms irrespective of their size have the propensity for distant metastases and should be regarded as carcinoma [294,295].

ONCOCYTIC ADENOMA Oncocytic adenoma is a completely encapsulated tumor with a distinct brown smooth and homogeneous cut surface; foci of hemorrhage and necrosis may be seen as secondary changes. On microscopic examination the tumor is composed of large polygonal cells with abundant finely granular eosinophilic cytoplasm arranged in a follicular pattern with areas of solid/trabecular growth. Predominance of a solid or trabecular pattern should raise the suspicion for malignancy and warrants careful evaluation of the capsule [132]. The nuclei of the tumor cells show a vesicular chromatin and may show significant atypia, hyperchromasia, and pleomorphism, which should not be mistaken as malignancy [132]. Foci of clear cell change may be seen, which is regarded as a degenerative phenomenon and sometimes the predominant lesion may have clear cell morphology. The tumor may show areas of ischemic necrosis that may sometimes involve the entire lesion, making histologic evaluation difficult [132]. This is most commonly seen following needle aspiration biopsy.

The distinction between oncocytic adenoma and carcinoma is based on the finding of capsular and/or vascular invasion as in the case of follicular tumors. This can sometimes be difficult and require extensive sampling of the capsule. Some studies have suggested that size is helpful in differentiating oncocytic adenoma from carcinoma, with tumors >4.0 cm being more likely to be malignant compared to the smaller tumors [296–298]. Other immunohistochemical markers such as Ki-67 and cyclin D1 expression have also been found to help in the differential diagnosis of oncocytic adenoma and carcinoma [299].

ONCOCYTIC CARCINOMA Oncocytic carcinoma is a rare malignant thyroid tumor that is more common in females, with a female-to-male ratio approaching 2:1, which is less than for papillary or follicular carcinoma (the female-to-male ratio for oncocytic adenoma is 8:1). Although it can occur at any age, it is most commonly seen in the elderly, which is a decade later than for the oncocytic adenoma [165]. Therefore, an oncocytic tumor in an elderly man, especially if it is >5.0 cm, should raise suspicion of malignancy.

On gross examination oncocytic carcinomas are larger than their benign counterparts, although in a wide size range, and show a brown cut surface with more marked areas of hemorrhage and necrosis compared to the adenoma. Histological examination most often shows a solid/trabecular growth pattern as opposed to a predominantly follicular pattern seen in adenoma, and although the hallmark of malignancy is the presence of capsular and vascular invasion as described earlier in follicular carcinoma, certain cytological features may be helpful. In carcinoma there is increase in the nuclear size, with a greater percentage of cells being tall columnar as opposed to the round or polygonal cells seen in adenoma [132]. In addition, there is more hyperchromasia and mitoses in carcinoma compared to adenoma. In some cases areas of clear cell change may be seen and these may sometimes predominate. Oncocytic carcinoma is also designated as *minimally invasive* or *widely invasive* using the same criteria as described earlier for follicular carcinoma. This distinction is important because of significant differences in the biologic behavior of the two groups. In one study, no patient with minimally invasive carcinoma died of disease after a median follow-up of 8 yr, whereas in the widely invasive carcinoma group 73% relapsed and 55% died of disease. Adverse prognostic factors include extrathyroidal extension, nodal metastases, and positive margin at surgery and solid growth pattern. Of these extrathyroidal extension and nodal metastases are independent predictors of prognosis on multivariate analyses [293].

Differential Diagnosis The diagnosis of oncocytic neoplasm in most cases is made by the distinctive oncocytic nature of the cells. Evaluation of the tumor capsule is needed to distinguish adenoma from carcinoma, as mentioned earlier in the case of follicular carcinoma. In the case of carcinoma, lesions that sometimes may have to distinguished include oncocytic variant of medullary carcinoma and parathyroid oxyphil tumor. If the tumor shows extensive clear cell change, it should be distinguished from other clear cell tumors such as metastatic renal cell carcinoma and clear cell medullary carcinoma. For medullary carcinoma, the presence of amyloid and positive immunohistochemical staining with chromogranin, calcitonin, calcitonin-gene-related peptide, and CEA are helpful.

PAPILLARY ONCOCYTIC NEOPLASMS Oncocytic tumors may sometimes exhibit a predominantly papillary architecture comprised of papillae without a well-developed fibrovascular core. These tumors are designated as oncocytic papillary neoplasms and should be distinguished from oncocytic papillary thyroid carcinomas based on the characteristic nuclear changes of papillary carcinoma [132].

Poorly Differentiated Carcinoma Differentiated thyroid carcinoma of both papillary and follicular type may progress to a more poorly differentiated phenotype. This poorly differentiated carcinoma may present as a more distinct morphologic entity referred to as insular carcinoma or a group of less-well-defined morphologic phenotype. The latter group of poorly differentiated thyroid carcinoma, which has included entities such as columnar and tall variants of papillary carcinoma and tumors with solid and trabecular architecture, has been controversial, with its inclusion as a distinct category in question [300].

INSULAR CARCINOMA Insular carcinoma is a morphologically distinct form of tumor derived from the thyroid follicular cells having an aggressive biological course. The incidence of these tumors varies from being rare in the United States to about 4% of all thyroid cancers seen in an Italian study [132]. We have ourselves seen several cases of pure insular carcinoma in our practice, and it is not uncommon to see foci of insular growth associated with differentiated thyroid tumors in some other cases (personal experience). These tumors tend to involve elderly patients with a slight female predilection [132].

On gross examination these tumors are large in size and exhibit an invasive growth with associated areas of necrosis (Fig. 12A). On histology they are composed of well-defined islands of round or oval cells that lack significant pleomorphism. Small microfollicular structures may be seen within these islands, and it is not uncommon to see necrosis in the center of these islands (Fig. 12B). Foci of vascular invasion are not infrequent [30,132].

Differential Diagnosis The tumor that insular carcinoma should be differentiated from and can pose problems on morphology is medullary carcinoma. Immunohistochemistry is very useful in these situations. Insular carcinoma cells are positive for thyroglobulin and negative for calcitonin and other neuroendocrine markers. Another primary tumor that may have to be

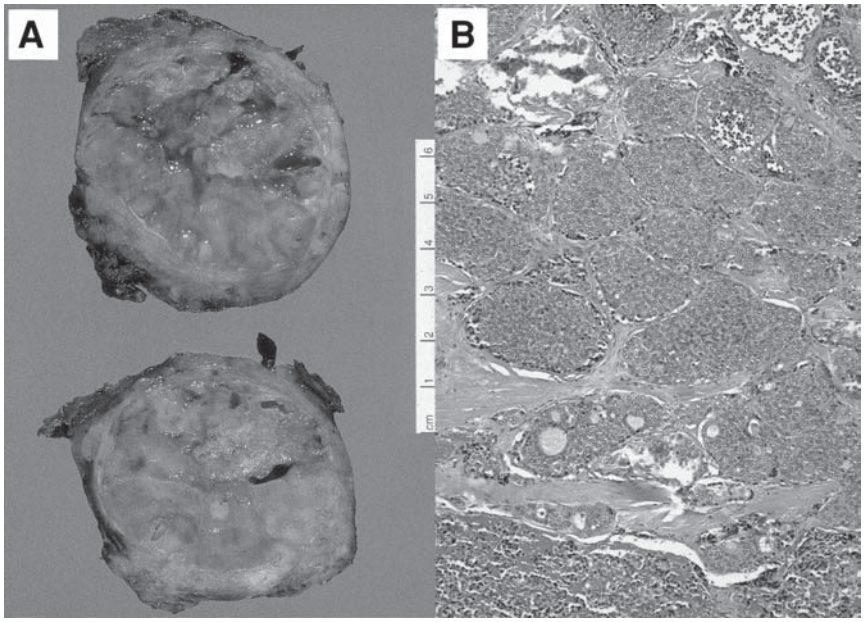

Figure 12 Insular carcinoma. (**A**) Macroscopic appearance of thyroid gland with insula carcinoma; (**B**) solid islands of tumor cells with microfollicular structure characteristic of insular carcinoma.

differentiated is the solid variant of papillary carcinoma, which is more common following exposure to nuclear radiation [270, 271]. In these cases the nuclear features of papillary carcinoma are helpful in differential diagnosis.

Anaplastic Carcinoma Anaplastic thyroid carcinoma is a highly aggressive and rare thyroid tumor, accounting for 1.7% of all thyroid cancers in the United States [165]. Its incidence is higher in regions endemic for goiter [301]. The patients are usually elderly in their seventh or eight decade of life, with a female preponderance of approx 2.5:1 [165]. A personal history of goiter may be seen in approx 25% of cases and prior exposure to radiation in 9.4% cases [165]. Patients present with rapidly enlarging neck mass associated with compressing symptoms such as dysphagia, hoarseness, and stridor. Often signs and symptoms related to metastatic tumor may be the first presentation [132,165]. On gross examination the tumor is usually large, the majority being >4.0 cm [165], often replacing the entire thyroid and spreading into the perithyroidal soft tissues [132]. On microscopic examination, the tumor may exhibit one of the three patterns or a mixture of more than one of these patterns including squamoid pattern resembling the nonkeratinizing squamous cell carcinoma, spindle cell pattern resembling sarcoma, and giant cell type (Fig. 13B). All three types of pattern are associated with a high mitotic rate and focal necrosis may be seen. The spindle cell sarcomalike pattern may show paucicellular areas with dense fibrosis that may alternate with cellular areas, which aids in the diagnosis of anaplastic thyroid carcinoma [30,132]. The giant cell pattern is associated with a marked degree of pleomorphism with large multinucleated cells including tumor giant cells. The tumors are highly invasive with infiltration of the perithyroidal soft tissues [132]. In some cases heterologous stromal elements including cartilage or bone may be seen [132].

On immunohistochemistry the squamoid-type tumors are positive for high- and low-molecular-weight keratin, EMA, and sometimes CEA. The spindle cell pattern shows variable positivity with low-molecular-weight keratin ranging in incidence from 47% to 100% cases. Thyroglobulin staining is also variable, ranging from 9% to 71% cases and is usually focal and weak [132]. Ultrastructurally evidence of epithelial differentiation is seen in most tumors [132].

DIFFERENTIAL DIAGNOSIS OF ANAPLASTIC CARCINOMA The squamoid type anaplastic carcinoma should be differentiated from *metastatic carcinoma* from primary sites such as lung, esophagus, and upper aerodigestive tract. The history of a rapidly growing mass in the region of the thyroid together with the finding of differentiated thyroid carcinoma in some areas and positive immunostaining with thyroglobulin may be helpful in the differential diagnosis.

The spindle-cell-type tumor should be differentiated from true *sarcoma* such as *fibrosarcoma, malignant fibrous histiocytoma, angiosarcoma*, and *hemangiopericytoma*. This may pose the greatest difficulty especially in cases where the entire tumor is of spindle cell type and is paucicellular. Features that favor anaplastic thyroid carcinoma include foci of better-differentiated areas, justifying thorough sampling of the tumor and evidence of epithelial differentiation on immunohistochemistry and electron microscopy. In addition, *metaplastic spindle cell proliferation*, which can sometimes be seen associated with papillary carcinoma and follicular adenoma [183], should also be differentiated from spindle-cell-type anaplastic carcinoma because of the significant difference in the biological behavior and management of the two entities.

Medullary thyroid carcinoma may show a variety of growth patterns, and this may have to be differentiated from the spindle cell type and pleomorphic variants of anaplastic carcinoma.

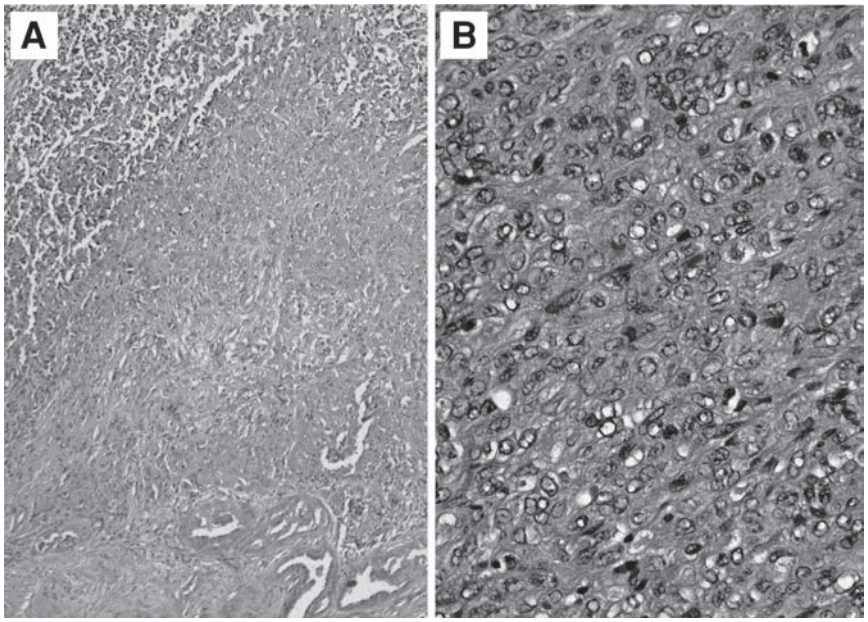

Figure 13 Anaplastic carcinoma. (**A**) Giant cell type with necrosis; (**B**) spindle cell type.

Immunohistochemistry plays a very important role in this differentiation and must be performed using antibodies to calcitonin, chromogranin, and CEA, which are all positive in medullary carcinoma [132].

Malignant lymphoma may also be considered in cases with small cells; however, the even distribution of smaller, uniform cells in lymphoma and lack of focal epithelial islands is helpful in this differential diagnosis [132].

Riedel's thyroiditis may be confused with the paucicellular spindle-cell-type anaplastic thyroid carcinoma, which may show marked sclerosis and lack significant pleomorphism and mitotic activity. In these cases, features that favor anaplastic carcinoma include the presence of necrosis, more cellular areas, and evidence of vascular invasion and metastases [30]. The more cellular areas and better differentiated thyroid carcinoma may be seen on extensive sampling, which is crucial in the diagnosis of anaplastic carcinoma in these problematic cases.

The prognosis in anaplastic thyroid carcinoma is extremely poor, with mean survival of 7.2 ± 10 mo [132].

Medullary Thyroid Carcinoma

Clinical Features and Etiology The tumor derived from the thyroid C cells that is now referred to as medullary carcinoma was first regarded as an unusual variant of anaplastic carcinoma [30]. The term medullary carcinoma was coined by Hazard et al. in 1959, who reviewed 600 cases of thyroid cancer over a 31-yr period at the Cleveland Clinic, reported 21 tumors with unique morphological features including solid growth pattern and amyloid stroma, and called them medullary carcinoma [302]. Later Williams in 1966 identified the cell of origin of these tumors as C cells [303]. Medullary thyroid carcinoma is a rare tumor with a incidence of 3.2% (0.5% familial and 2.7% sporadic) of all thyroid cancers in a cohort of more than 5500 patients in the United States [165]. One of the unique features of this tumor is that it occurs both in a familial setting associated other endocrine neoplasms as part of the multiple endocrine neoplasia syndrome (MEN) and also as sporadic tumors in a nonfamilial setting [304,305].

Sporadic medullary carcinoma is the more common form of tumor, representing approx 80% of all medullary carcinomas. The remaining 20% are familial and are inherited as autosomal dominant trait with high penetrance [30,165]. The latter may either occur in association with other endocrine tumors as part of MEN 2A, which includes in addition to medullary carcinoma, adrenal medullary hyperplasia–pheochromocytoma and parathyroid hyperplasia–adenoma and MEN 2B [3], which includes, in addition to pheochromocytoma, mucosal ganglioneuroma and skeletal abnormalities. In addition these may occur as familial non-MEN medullary thyroid carcinoma [306–309]. The gene for MEN 2A and 2B syndrome is called the *RET* protooncogene, which is located on chromosome 10. Germline missense mutation of this gene is seen in patients with MEN 2A and MEN 2B [306,307,310–312]. Sporadic tumors may sometime show mutation in the *RET* protooncogene [312].

The age at presentation in the familial form of medullary carcinoma is younger than in the sporadic form and both show a slight female predilection [132]. With increased prospective screening of family members of MEN patients, the age at presentation of familial form of medullary carcinoma is becoming progressively younger [132]. The sporadic form presents with a solitary mass with or without lymph node metastases. The familial form is usually multicentric and bilateral. In addition, patients may have diarrhea and both carcinoid and Cushing's syndromes have been reported [132,304,305]. Increased levels of serum calcitonin and CEA are seen in both familial and sporadic medullary carcinoma and are important diagnostic tests [132].

Pathology of Medullary Carcinoma The tumors vary in size from barely visible to large tumors that may replace the entire lobe; the smaller tumors are often seen in patients with MEN 2 syndrome who undergo prophylactic thyroidectomy for high serum calcitonin levels that are discovered at routine

screening. The small tumors, which are <1.0 cm, are referred to as medullary microcarcinoma and are commonly seen at the junction of upper and middle thirds of the lobes [304,313–315]. In these cases it is important that the entire gland be submitted so that small tumors that may not be apparent grossly are not missed. On sectioning the tumors have firm yellow white cut surface. The large tumors usually have an indistinct border and most are not encapsulated. However, rarely a thick fibrous capsule may sometimes be seen and may sometimes be associated with cystic and papillary change [132,316].

On microscopic examination medullary carcinomas are usually circumscribed and show a variety of growth patterns that may mimic follicular, papillary, insular, and anaplastic thyroid carcinoma, emphasizing the importance of careful morphologic evaluation and use of immunostaining to differentiate it from these follicular-cell-derived tumors [30,132,317]. The most typical growth pattern includes solid nests or trabeculae or an insular pattern separated with thin fibrovascular core. The cells within these nests are round, oval, or spindle shaped, with a finely granular dispersed chromatin typical of neuroendocrine cells; giant multinucleated cells may sometimes be seen. The cytoplasm is amphophilic or eosinophilic and may appear clear, sometimes with occasional mucin positive intracytoplasmic vacuoles [132]. Amyloid (Fig. 14F) may be seen in the stroma in up to 80% of tumors, and stains positively with calcitonin on immunohistochemistry. In the familial type of medullary carcinoma areas of C-cell hyperplasia are usually seen associated with the tumor, which is a feature not seen in sporadic tumors [132,296,318]. On immunohistochemistry, the tumor cells are negative for thyroglobulin and positive for panneuroendocrine markers such as synaptophysin and chromogranin, and more specific markers such as calcitonin and calcitonin gene-related peptide. In addition, CEA is another useful marker that is positive in medullary carcinoma [132,317]. Sex steroid receptors, especially the progesterone receptor, may be seen in medullary carcinoma on immunohistochemistry [319]. Ultrastructurally the characteristic membrane-bound neurosecretory granules are seen in the cytoplasm [132].

Medullary carcinoma variants include *follicular* variant, *papillary* variant, *oncocytic* variant (Fig. 14E), *small cell* variant, *giant cell* variant, *clear cell* variant, *melanotic* variant, *squamous* variant, *encapsulated* variant, and *paraganglioma-like* variant [132,316,320]. The presence of all these variants makes medullary carcinoma the great mimicker of other thyroid carcinoma; therefore immunohistochemistry should be performed in all cases when the diagnosis is in doubt or some atypical features are present.

The prognosis in medullary carcinoma shows considerable variation. The 5- and 10-yr survival rates are 60–70% and 40–50%, respectively. Better prognostic factors include younger age, females, early tumor stage, and familial tumors [132,321].

C-Cell Hyperplasia and Medullary Microcarcinoma

C-Cell Hyperplasia

General Considerations The calcitonin-producing C cells are derived from the neural crest and descend down into the thyroid with the ultimobranchial body; therefore, they are found mainly at the junction of the upper and middle third of the two thyroid lobes, frequently associated with solid cell nests [14]. C cells are usually visualized on routine hematoxylin and eosin stains but may be easily seen on immunohistochemistry (Fig. 14). The increase in the numbers of C cells is referred to as C-cell hyperplasia. C-cell hyperplasia, which was first described by Wolfe in 1973 in patients with MEN 2A [322], can also be seen to be associated in certain other situations with no evidence of MEN 2. Whereas the former is referred to as familial C-cell hyperplasia, thought to be a neoplastic proliferation, the latter is called physiological or secondary C-cell hyperplasia [323,324].

The *familial C-cell hyperplasia* is thought to be neoplastic and is regarded as a precursor of familial type of medullary carcinoma; therefore the term hyperplasia for these lesions is a misnomer and has been questioned [323,325]. It is thought to represent the preinvasive stage (carcinoma *in situ*) of medullary carcinoma [324]. The neoplastic nature of this form of "hyperplasia" is further supported by the fact that C cells are clonal [326] and show a polysialic acid immunostaining pattern that is distinct from the physiological/reactive C-cell hyperplasia [327].

Physiological C-cell hyperplasia, also referred to as reactive or secondary C-cell hyperplasia, has been associated with conditions causing hypercalcemia such as hyperparathyroidism [328,329]. This form of C-cell hyperplasia has also been seen associated with Hashimoto's thyroiditis [330,331], multinodular goiter, hyperthyroidism, lymphoma [332,333], around thyroid tumors of follicular cell origin [334], and following subtotal thyroidectomy [335].

Diagnosis The diagnostic criteria for what constitutes C-cell hyperplasia are not clearly established. It includes definitions ranging from imprecise descriptions of C-cell volume, which can be very subjective [323,336], to semiquantatative estimates such as >50 cells per 50 low-power fields to >50 cells per 1 low-power field [323]. Guyent et al. have proposed more precise quantitative criteria that include >40 cells/cm^2 or >50 cells in 3 low-power (×100) fields [337]. We believe that for practical purposes if one can see C cells on routine hematoxylin and eosin stain that are later confirmed as C cells on immunohistochemistry, this should be considered C-cell hyperplasia (Fig. 14A,B). In patients with MEN 2 who undergo thyroidectomy for rising serum calcitonin levels, the entire thyroid gland should be submitted to identify areas of C-cell hyperplasia and immunohistochemistry should be performed. C-cell hyperplasia can be focal, diffuse, or nodular depending on the extent of follicular involvement and morphology [132]. On immunohistochemistry, C cells stain positively for chromogranin, synaptophysin, calcitonin, calcitonin gene related peptide, and CEA [132].

Differential Diagnosis C-cell hyperplasia should be distinguished from solid cell nests, islands of squamous metaplasia that can be seen in Hashimoto's thyroiditis or in remnants of branchial clefts, intrathyroidal remnants of thymus and parathyroid tissue, and finally follicular cells appearing as solid clusters as a result of tangential sectioning. Immunohistochemistry can be very useful to distinguish these conditions from C-cell hyperplasia. However, one important lesion that has to be distinguished from nodular-type C-cell hyperplasia and can be challenging sometimes is a small medullary microcarcinoma described below.

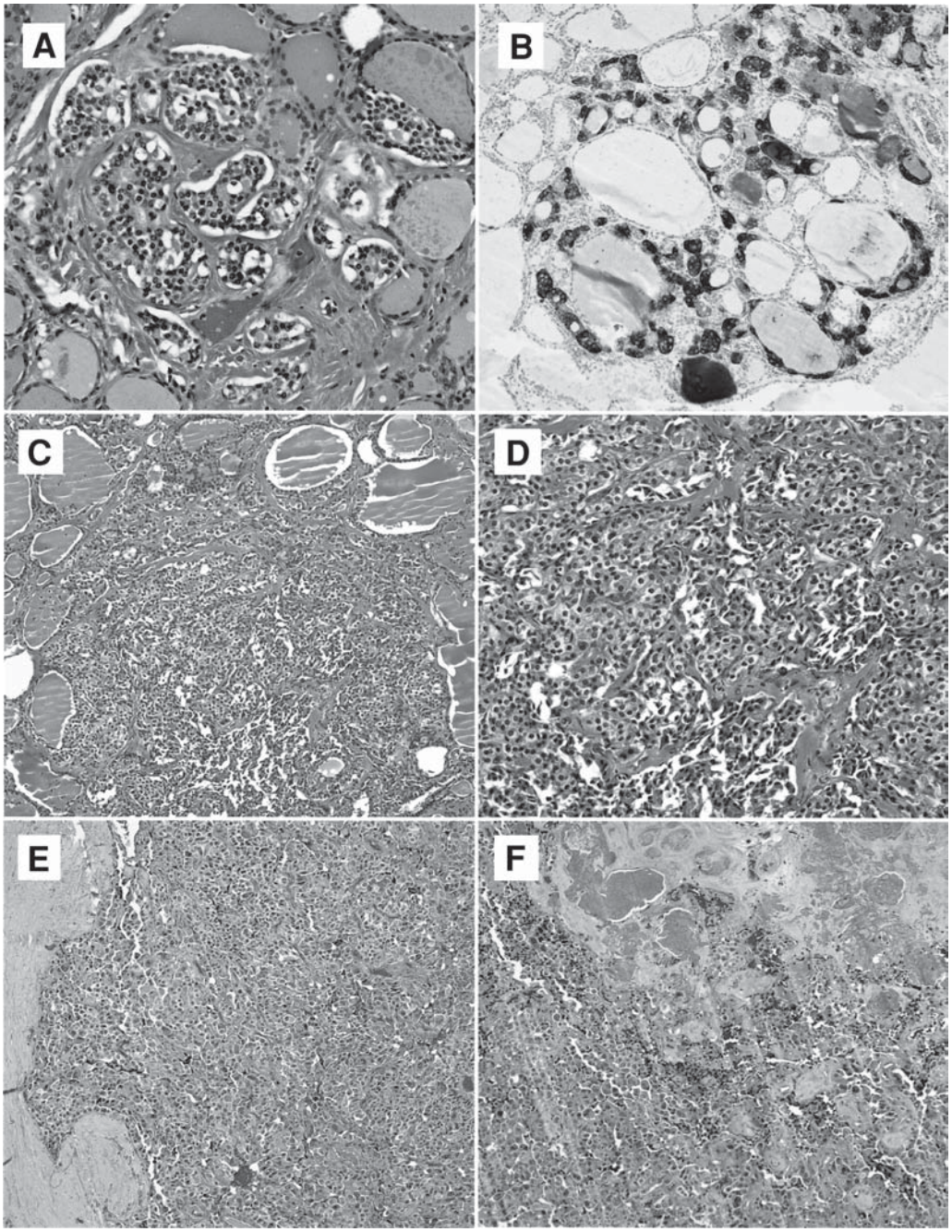

Figure 14 C-cell hyperplasia and medullary carcinoma. (**A**) C-cell hyperplasia (H&E); (**B**) C-cell hyperplasia showing positive immunostaining with calcitonin stain (immunoperoxidase); (**C**) medullary microcarcinoma in a familial case associated with C-cell hyperplasia; (**D**) same case as in **C** showing central portion of the medullary microcarcinoma; (**E**) medullary carcinoma, oncocytic variant; (**F**) same tumor as in **E** showing amyloid in the stroma. (Color illustration appears in insert following p. 148.)

Medullary Microcarcinoma

General Considerations Medullary thyroid microcarcinoma is defined as a tumor <1.0 cm in size. Its frequency is directly related to screening of MEN 2 family members using genetic testing for *RET* proto-oncogene mutation and is likely to increase with more widespread genetic testing [323]. Medullary microcarcinoma can be seen both in familial setting and also as sporadic tumor.

The *sporadic medullary microcarcinoma* are more likely to be unilateral and solitary and most of the time are identified either as an incidental finding in thyroidectomy specimens or at autopsy or by elevated serum calcitonin levels. Rarely, these tumors can be symptomatic and the symptoms may include diarrhea, palpable mass, enlarged cervical lymph nodes [313,315,323, 338], or lung metastases as seen in one series [313]. In view of the high incidence of lymph node metastases, aggressive surgical management has been proposed in these patients [314]. Adverse prognostic factors include symptomatic cases, high serum calcitonin level, amyloid stroma, and desmoplasia [304,313].

Familial medullary microcarcinoma are seen in patients showing germline mutation of the *RET* proto-oncogene They are detected either on genetic testing or on the finding of high

serum calcitonin levels. They are usually bilateral and unilateral tumors are more likely to be multifocal. Small tumors may be overlooked on gross examination, therefore the entire thyroid should be submitted starting from superior to inferior poles of both lobes for identification of these tumors [323]

Pathology of Medullary Microcarcinoma and Differential Diagnosis If the tumors are very small, they may not be grossly identified; lesions that are visible grossly are firm, whitish nodules commonly situated in the upper or middle third of the lobe [313]. On microscopic examination the tumors commonly show trabecular, solid, lobular/diffuse growth pattern. Follicular and pseudopapillary pattern may also be seen but are rare and usually associated with one of the above patterns. The cells are round or fusiform with finely dispersed chromatin [313, 323]. Stromal fibrosis and amyloid deposition may be seen but is usually less than what is seen in sporadic medullary carcinoma >1.0 cm in size [323]. Familial medullary microcarcinoma are usually associated with C-cell hyperplasia (Fig. 14C,D). One of the main differential diagnoses of medullary microcarcinoma is nodular C-cell hyperplasia. The feature that favors medullary carcinoma is the breach of the basement membrane and associated desmoplasia [317,339]. Some authors have used collagen IV immunostaining for evaluation of the basement membrane [340].

Prognosis of Medullary Microcarcinoma The prognosis in familial medullary microcarcinoma is much better than for larger medullary carcinoma. None of the patients in four series reviewed by Albores-Saavedra and Krueger [318,323] died of tumor, with follow-up being up to 20 yr in one of the series. In sporadic medullary microcarcinoma, none of the patients in a series of 34 patients by Kaserer et al. [304] showed evidence of local recurrence or mortality during the mean follow-up of 27 mo. However, in one other series of 38 patients, two patients died of tumor after 24 and 46 mo [313]. Beressi et al. [314] reported a survival rate of 93.9% at 10 yr, which was significantly greater than what was seen in sporadic medullary carcinoma >1.0 cm.

Mixed Medullary and Folloicular Carcinoma These tumors show mixed morphology including both medullary and follicular patterns and mixed immunoreactivity with thyroglobulin and calcitonin [341–343]. In view of this dual differentiation some have proposed the idea of common stem cell origin for thyroid cells similar to that seen in the gastrointestinal tract [1]. Composite tumors are referred to as tumors with two distinct cell populations, one thyroglobulin positive and the other of C-cell derivation which stains with calcitonin on immunohistochemistry [30]. Immunohistochemistry should be performed for the diagnosis of these mixed tumors and will also aid in differentiating these tumors from progression of a differentiated tumor with follicular architecture to an insular poorly differentiated phenotype.

Other Rare Epithelial Thyroid Tumors

MUCOEPIDERMOID CARCINOMA Primary mucoepidermoid carcinoma (MEC) of the thyroid is rare but has generated interest among pathologists with respect to its cell of origin, which has included solid cell nests, follicular cells, and even C cells. On histologic examination some thyroid MEC may show marked stromal sclerosis and tissue eosinophilia in addition to the characteristic squamoid and mucin-producing areas as seen in these tumors in other locations [30,344–348]. The presence of marked tissue eosinophilia and sclerosis may mimic Hodgkin's disease in lymph node metastases of these tumors and should be considered in the differential diagnosis [349].

SQUAMOUS CELL CARCINOMA Primary squamous cell carcinoma of the thyroid is rare; spindle cell squamous cell carcinoma has been described in association with tall cell variant of papillary carcinoma and is regarded as an unusual type of anaplastic carcinoma [350].

SPINDLE EPITHELIAL TUMOR WITH THYMUSLIKE DIFFERENTIATION (SETTLE) This is a rare thyroid tumor, which occurs predominantly in children and young adults. These tumors are slow growing and present as painless thyroid masses. Local recurrence and distant metastases involving lungs may be seen later in the course of the disease [351–355]. SETTLE is thought to be derived from a thymic or branchial pouch remnant [352]. On microscopic examination, the tumor shows lobules of tumor cells separated by fibrous bands; the tumor cells are spindle shaped mixed with tubulopapillary epithelial islands including foci of squamoid areas and structures resembling Hassal's corpuscles. Cystic change may be seen in the epithelial islands [352–354]. On immunohistochemistry the tumor cells stain positive with cytokeratin and muscle-specific actin and they are negative for thyroglobulin and calcitonin [353].

Differential Diagnosis Differential diagnosis of thyroid spindle cell lesions includes anaplastic carcinoma, medullary carcinoma, intrathyroidal thymoma, metaplastic spindle cell proliferation associated with follicular-cell-derived tumors, teratoma of the thyroid, synovial sarcoma, and other mesenchymal tumors. The age at presentation in SETTLE (children) helps rule out anaplastic carcinoma and intrathyroidal thymoma. Positive immunoreactivity with cytokeratin rules out mesenchymal tumor except synovial sarcoma. Medullary carcinoma and synovial sarcoma both may be seen in childhood, medullary carcinoma will stain positive for neuroendocrine markers on immunohistochemistry, and synovial sarcoma is usually highly mitotic and lacks cyst formation [30].

CARCINOMA SHOWING THYMUSLIKE DIFFERENTIATION (CASTLE) This is a rare tumor first described by Miyauchi et al. in 1985 [356] and has also been referred to as intrathyroidal thymoma (ITET). It occurs in the elderly, involves the lower or middle third of the gland, and may invade surrounding soft tissues and regional lymph nodes. On histology it shows a lobulated architecture composed of groups of tumor cells with large vesicular nuclei, prominent nucleoli, and associated prominent lymphocytic infiltrate reminiscent of the so-called lymphoepithelioma [355]. On immunohistochemistry tumor cells are positive for CD5, suggesting a thymic origin; they are also positive for cytokeratin and both thyroglobulin and calcitonin are negative. Differential diagnosis of CASTLE includes metastatic carcinoma, particularly from the lung and upper aerodigestive tract [132]. CD5 immunoreactivity is useful in this differential diagnosis, as most metastatic carcinoma from the sites mentioned above are CD5 negative [356].

Rare Nonepithelial Thyroid Tumors

LYMPHOMA Thyroid lymphoma is rare and accounts for 2.2–2.5% of all lymphomas [30]. It usually occurs in the background of Hashimoto's thyroiditis and is thought to arise from

the mucosa associated lymphoid tissue (MALT). The most common type of lymphoma is the diffuse large B-cell lymphoma followed by low-grade MALT lymphoma. Diffuse large B-cell lymphoma must be differentiated from anaplastic carcinoma and immunohistochemistry can be very helpful in this situation [357,358].

OTHER NONEPITHELIAL TUMORS Mesenchymal tumors, which are more commonly seen in other parts of the body, are rare in the thyroid. Primary mesenchymal tumors that have been described in the thyroid include vascular tumors such as cavernous hemangioma [359] and epithelioid hemangioendothelioma [360], granular cell tumor [361], solitary fibrous tumor [362,363], and smooth muscle tumors including leiomyoma and leiomyosarcoma [30].

Other tumors and tumorlike lesions that have described in thyroid include Langerhans' cell histiocytosis [364], plasma cell granuloma [365], and extramedullary hematopoesis [366].

Metastatic Carcinoma Metastasis to thyroid from another primary source is infrequent. Tumors that most commonly spread to the thyroid include kidney, breast, and lung carcinoma [132]. Metastatic carcinoma should be considered in the differential diagnosis of poorly differentiated carcinoma, especially with a noninsular growth pattern, and clinicopathological correlation and immunostaining with thyroglobulin may be helpful in these situations [367,368].

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10 Recent Developments in the Molecular Biology of the Thyroid

YURI E. NIKIFOROV, MD, PHD

INTRODUCTION

The last decade has seen a significant expansion in our understanding of the molecular biology of the thyroid. It has become clear that the molecular landscape of thyroid tumors arising from follicular epithelium is dominated by chromosomal rearrangements, which are otherwise prevalent in hematologic malignancies and sarcomas, but not in other types of epithelial neoplasms. The identification of the gene responsible for the familial forms of medullary carcinomas, originated from thyroid C-cell tumors, has led to a dramatic change in the management of patients with this disease, and is one of the first examples of preventive surgery performed solely on the basis of molecular genetic testing. The progress in molecular biology is expected to permeate into virtually all aspects of thyroid pathology and provide significant assistance in the diagnosis of thyroid tumors, in the determination of tumor prognosis, as well as to serve as an additional aid for proper classification of thyroid tumors. In this respect, it is important to realize that the thyroid gland represents a unique model of tumorigenesis, as thyroid follicular cells give rise to the malignant tumors with a widely variable biological behavior. Indeed, well-differentiated papillary, follicular, and Hürthle cell carcinomas have an overall favorable prognosis; poorly differentiated carcinoma behaves in a more aggressive manner, while anaplastic carcinoma is one of the deadliest human malignancies. In this chapter, we follow the general classification of thyroid tumors and summarize molecular alterations identified in each type of thyroid malignant and benign neoplasms.

PAPILLARY THYROID CARCINOMA

Papillary carcinoma is the most common malignant thyroid tumor, accounting for approx 80% of all thyroid cancers [1]. It has a distinct propensity for invasion of lymphatic channels, resulting in a high incidence of multifocal involvement of the thyroid gland and regional cervical lymph node metastases [2]. Distant blood-borne metastases are uncommon (5–10%); about 10% of tumors recur locally. Overall, papillary carcinoma has the best prognosis of all thyroid malignancies, with an average 93% 10-yr survival rate in the United States [3]. However, its behavior varies widely, from small tumors with little evidence of invasion found incidentally at autopsy, to rapidly growing and widely invasive tumors that metastasize and cause patient deaths. Multiple attempts have been made to predict tumor behavior based on microscopic features and more recently on molecular alterations. A number of genetic abnormalities have been identified in papillary carcinomas, most of which are large-scale chromosomal rearrangements involving the *RET* and *TRK* genes, while point mutations of *RAS* and genomic instability are present at a much lower rate.

RET/PTC REARRANGEMENT

Structure and Function of *RET/PTC* Oncogene Activation of the *RET* gene by rearrangement is the most common genetic event identified to date in papillary carcinomas. The *RET* protooncogene is located on chromosome 10q11.2 and encodes a cell-membrane-receptor tyrosine kinase [4,5]. It is normally expressed primarily in neural crest–derived cells and in urogenital precursor cells during embryogenesis, and plays an important role in the development and survival of these cell lineages [6]. Like other tyrosine kinase receptors, it consists of an extracellular domain, a transmembrane domain, and an intracellular domain that includes a region with protein-tyrosine kinase activity (Fig. 1A). The ligands for *RET* are neurotrophic factors of the glial cell line–derived neurotrophic factor (GDNF) family, including GDNF, neurtulin, artemin, and persephin [7, 8]. Ligand binding, mediated by cell-surface-bound coreceptors GFRα, leads to *RET* dimerization and autophosphorylation on tyrosine residues, which initiates the activation of downstream signaling pathways. In the thyroid gland, wild-type *RET* is expressed at a high level in parafollicular C cells, but not in thyroid follicular cells, where it can be activated by chromosomal rearrangement named *RET/PTC* (PTC for papillary thyroid carcinoma).

As a result of the rearrangement, the *RET* gene is separated into two parts, and its intracellular tyrosine kinase domain is fused with the 5' terminal sequence of different unrelated genes. Three types of *RET/PTC* were originally identified and remain by far the most common in papillary carcinomas (Fig. 1B). Of these, *RET/PTC1* is formed by fusion with the *H4* (also known as *D10S170*) gene [9], and *RET/PTC3* by fusion with the *ELE1* (*RFG* or *ARA70*) gene [10,11]. *RET/PTC1* and *RET/PTC3* are

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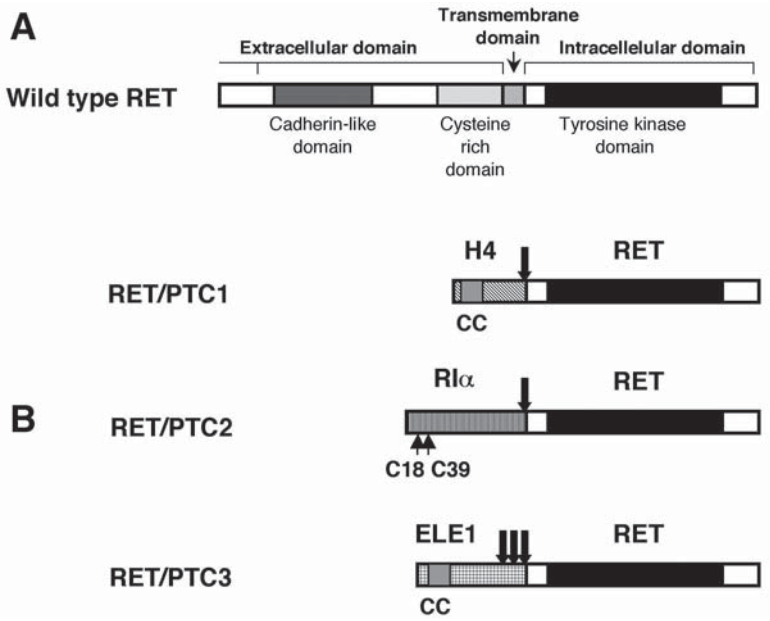

Figure 1 (**A**) Schematic representation of the wild-type *RET* gene that encodes a receptor tyrosine kinase. Its extracellular domain contains a region of homology with cadherins and a juxtamembrane cysteine-rich region. The intracellular domain has a region with protein-tyrosine kinase activity, which functions in phosphorylation of key tyrosine residues, and is involved in interaction with downstream targets and activation of signaling pathways. (**B**) Schematic representation of three major types of *RET/PTC*, formed by fusion of the intracellular domain of RET to the 5' portions of the *H4*, *RIα*, or *ELE1* gene. The genes fused with *RET* encode dimerization domains, either coiled-coil domain (CC) or cysteine residues forming disulfide bonds during dimerization (C18, C39), allowing ligand-independent dimerization and activation of the truncated *RET* receptor. *Block arrows* indicate breakpoint sites.

Table 1
Characteristics of the Different Types of *RET/PTC* Rearrangement in Papillary Thyroid Carcinoma

Type of RET/PTC	*Gene fused with RET*	*Mechanism of rearrangement*	*Prevalence among all RET/PTC types*	*Association with radiation exposure*
RET/PTC1	*H4 (D10S170)*	inv(10)(q11.2;q21)	60–70%	Some cases
RET/PTC2	*RIα*	t(10;17)(q11.2;q23)	<10%	Some cases
RET/PTC3 (and *RET/PTC4*)	*ELE1 (RFG, ARA70)*	inv(10)(q11.2)	20–30%	Strong
RET/PTC5	*GOLGA5*	t(10;14) (q11.2;q?)	Rare	Yes
RET/PTC6	*HTIF1*	t(7;10)(q32;q11.2)	Rare	Yes
RET/PTC7	*FRG7*	t(1;10)(p13;q11.2)	Rare	Yes
RET/ELKS	*ELKS*	t(10;12)(q11.2;p13)	Rare	No
RET/KTN1	*KTN1*	t(10;14)(q11.2;q22.1)	Rare	Yes
RET/RFG8	*RFG8*	t(10;18)(q11.2;q21–22)	Rare	Yes
RET/PCM-1	*PCM-1*	t(8;10)(p21–22;q11.2)	Rare	Yes

intrachromosomal paracentric inversions, as both genes participating in the rearrangement are located on chromosome 10q [12,13]. In contrast, *RET/PTC2* is formed by a reciprocal translocation between chromosomes 10 and 17, resulting in *RET* fusion with the regulatory subunit RIα of the cAMP-dependent protein kinase A [14]. Recently, seven novel types of *RET/PTC* have been described in single cases of radiation-induced (*RET/PTC5, RET/PTC6, RET/PTC7, RET/KTN1, RET/RFG8, RET/PCM-1*) and sporadic (i.e., not associated with radiation) (*RET/ELKS*) papillary carcinomas [15–20]. All of these result from the fusion of the tyrosine kinase domain of *RET* with the genes located on different chromosomes (Table 1).

The genes fused with *RET* are constitutively expressed in thyroid follicular cells and drive the expression of the chimeric *RET/PTC* oncogene. In addition, these partners provide a dimerization domain that is essential for activation of the *RET* tyrosine kinase in the absence of a ligand [21,22]. In fact, all *RET* fusion genes encode putative dimerization domains, typically one or more coiled-coil domains [22]. Another important function of the genes fused with *RET* is in determining a subcellular localization of the chimeric *RET/PTC* protein, which lacks the transmembrane domain and cannot be anchored to the cell membrane. In *RET/PTC3* protein, for example, the N-terminal coiled-coil domain of ELE1 (RFG) not only mediates the dimerization of the receptor and chronic kinase activation, but is also responsible for the compartmentalization of the chimeric protein at plasma membrane level where most of the normal ELE1 (RFG) protein is distributed [23]. Thus, different types of *RET/*

PTC protein, which have a similar *RET* tyrosine kinase domain but different N-terminal portions, are likely to be distributed in various cytoplasmic compartments and interact with different substrates. This may explain some variations in phenotypes and biological properties recently found in tumors carrying *RET/PTC1* and *RET/PTC3* oncogenes.

RET/PTC is tumorigenic in thyroid follicular cells. This has been demonstrated by its ability to transform thyroid cells in culture [24], and to give rise to thyroid papillary carcinomas in transgenic mice. Indeed, most of the transgenic animals with targeted thyroid expression of *RET/PTC1* [25,26] and *RET/PTC3* [27] develop thyroid tumors with cytological features of human papillary carcinoma, including irregularly shaped nuclei, intranuclear inclusions, and grooves. It has been suggested that *RET/PTC* may be directly responsible for these characteristic nuclear features [28]. Thus, primary cultured human thyroid cells transfected with *RET/PTC1* retroviral construct appeared to acquire open chromatin, irregularity of nuclear contours, and occasional nuclear grooves and pseudoinclusions [28]. This effect was not noted in primary cultures transfected with the retroviral construct only, suggesting that this is a direct effect of *RET/PTC* expression.

Prevalence of *RET/PTC* in Thyroid Tumors *RET/PTC* rearrangements have so far been found only in thyroid lesions, and are generally believed to be restricted to the papillary type of thyroid carcinoma [29]. In the original study of 177 papillary carcinomas, 37 follicular, 15 anaplastic, 18 medullary carcinomas, and 34 benign adenomas by Southern blot analysis, *RET/PTC* was detected in 19% of papillary carcinomas, but not in other malignant and benign tumors [30]. More recently, these findings were confirmed in a series of 316 thyroid tumors where 40% of 201 papillary carcinomas, but none of 22 follicular carcinomas, 15 poorly differentiated carcinomas, 17 anaplastic carcinomas, or 61 follicular adenomas demonstrated evidence of *RET/PTC* by immunohistochemistry and in some cases by reverse transcriptase–polymerase chain reaction (RT-PCR) [31].

Recently, two studies have reported the occurrence of *RET/PTC* in hyalinizing trabecular adenomas [32,33]. In one observation, four tumors showed *RET* expression by immunohistochemistry and three of those were found to harbor *RET/PTC1* rearrangement by RT-PCR [32]. In another study, *RET/PTC1* was detected in six out of eight hyalinizing trabecular adenomas by RT-PCR [33]. It has been suspected for a long time that hyalinizing trabecular adenoma is related to papillary carcinoma, as it shares with it several cytologic features, as as nuclear grooves and pseudoinclusions; may contain psammoma-like calcifications; and could be seen in some cases merging with papillary carcinoma [34]. The recent molecular findings provide an additional evidence suggesting that hyalinizing trabecular adenomas may represent a peculiar variant of papillary carcinoma.

The presence of *RET/PTC* in follicular adenomas, hyperplastic thyroid nodules, and Hashimoto's thyroiditis, reported in some observations [35–40], has not been confirmed in other studies and remains controversial (reviewed in [41]].

In papillary carcinomas, the prevalence of *RET/PTC* shows a wide variation between different studies and geographic regions. In the United States, the five largest series reported the frequency ranging from 11% to 43%, with a total of 134 (35%) positive cases out of 386 papillary carcinomas cumulatively studied [30,31,42–44]. A comparable rate has been reported by other groups with a long-standing interest in the field from Canada (40%) [45] and Italy (29–35%) [30,46,47]. In other regions, a wide variation in frequency of *RET/PTC* has been reported, ranging from 3% in Saudi Arabia [48] to 85% in Australia [49]. Apart from the geographic variability, which clearly exists, some differences are likely due to the variation in screening techniques. Most studies reporting a very high incidence of the rearrangement were based on RT-PCR, the approach that offers the highest sensitivity, offset by a higher susceptibility to contamination and PCR-related artifacts and therefore to false-positive results. Other techniques such as Southern blot, fluorescence *in situ* hybridization (FISH), and *in situ* hybridization (ISH), are slightly less sensitive and technically more challenging, but carry a minimal risk of false-positive results (reviewed in [41]).

A higher prevalence of *RET/PTC* has been found in papillary thyroid carcinomas from children and young adults [46, 50–52]. Thus, in a series of 92 papillary carcinomas from Italy, 67% of tumors from patients aged 4–19 yr harbored *RET/PTC*, in contrast to 32% in those 31–80 yr old [46]. In the US series, it was seen in 45–71% of papillary carcinomas from young patients [50,51].

Among the different types of rearrangement, *RET/PTC1* is typically the most common and comprises up to 60–70% of positive cases, whereas *RET/PTC3* accounts for 20–30%, and *RET/PTC2* for fewer than 10% [31,44,47]. The novel types of *RET/PTC* are rare (Table 1).

The prevalence of *RET/PTC* is significantly higher in papillary carcinomas from patients with a history of radiation exposure, including those subjected to either accidental or therapeutic external irradiation. Among papillary carcinomas from children affected by the Chernobyl nuclear accident, 67–87% of tumors removed 5–8 yr after exposure and 49–65% of those removed 7–11 yr after the accident harbored *RET/PTC* [50,53–56]. Interestingly, *RET/PTC3* was the most common type in tumors developed <10 yr after exposure, whereas those removed after the longer latency had predominantly *RET/PTC1* [55,56]. In patients subjected to therapeutic external irradiation for benign or malignant conditions, a 52–84% prevalence has been reported [36,40]. Radiation exposure not only leads in a higher incidence of *RET/PTC* in papillary carcinomas, but also promotes the fusion of *RET* to unusual partners, as six out of seven novel *RET/PTC* types were detected in tumors associated with radiation exposure, where they constituted up to 4% of all rearrangements [16,18,19,57,58]. The prevalence of *RET/PTC* in different populations is summarized in Table 2.

The association between *RET/PTC* and ionizing radiation is supported by several studies demonstrating the induction of the rearrangement by in vitro irradiation of human undifferentiated thyroid carcinoma cells [59] and of fetal human thyroid tissues transplanted into severe combined immunodeficiency disease (SCID) mice [60,61]. The potential mechanism of how radiation may induce *RET/PTC* has been proposed. It appeared that chromosomal regions containing the *RET* and *H4* genes, participating in *RET/PTC1* rearrangement, are frequently juxta-

Table 2
Prevalence of *RET/PTC* Rearrangements in Papillary Carcinomas in Various Populations

	Prevalence	*Most common type*
Adults,		
general population	30–40%[a]	*RET/PTC1*
Children,		
general population	50–60%	*RET/PTC1*
Post-Chernobyl	60–70%[b]	*RET/PTC3*[b]
External therapeutic irradiation	60–70%	*RET/PTC1*

[a]In the United States, Canada, and Italy; prevalence widely varies in different geographic areas.
[b]In tumors developed <10 yr after exposure.

posed to each other in the nuclei of normal thyroid follicular cells [62]. Such spatial proximity may predispose the two chromosomal regions to simultaneous damage by radiation, possibly by a single radiation track, and facilitate mis-rejoining of free DNA ends located immediately adjacent to each other, which would result in the generation of *RET/PTC*.

Correlation with Microscopic Variants of Papillary Carcinoma *RET/PTC* rearrangements have been found in papillary carcinomas with classic papillary architecture and in different microscopic variants of the tumor. Overall, this rearrangement, and especially the *RET/PTC1* type, appears to be more common in tumors with pure or predominantly papillary growth and in papillary microcarcinomas (<1 cm in size) [31,63,64]. In some studies, the follicular variant of papillary carcinoma had slightly lower prevalence (10–26%) as compared to classic papillary carcinoma (43–47%) [31,52]. *RET/PTC* has also been reported in the diffuse sclerosing variant [47,50], tall cell variant [65], columnar cell variant [47], Hürthle cell papillary thyroid carcinomas [66], and in familial adenomatous polyposis-associated papillary thyroid carcinomas which have a peculiar cribriform/solid growth pattern [67,68].

A clear correlation between different *RET/PTC* types and morphological variants of papillary carcinoma has been observed in tumors from children exposed to radiation after the Chernobyl accident. In these populations, the solid variant of papillary carcinoma had a strong correlation with *RET/PTC3* and classic papillary carcinoma with *RET/PTC1* (Fig. 2). This finding, originally reported in a series of 38 post-Chernobyl papillary carcinomas [50], has been more recently confirmed in two larger series of post-Chernobyl tumors [56,69]. It remains unclear, however, if such a phenotype–genotype correlation exists in the general population also. It has not been found in a recent study of sporadic solid variant and classic papillary carcinomas, although the number of cases analyzed for *RET/PTC* was small [70].

Recently, a strong association between *RET/PTC3* and the tall cell variant of papillary carcinoma was reported [65]. In a large series of tumors studied for *RET/PTC1* and *RET/PTC3*, both types were almost equally common in classic papillary carcinomas and its follicular variants, whereas among 39 tall cell variants, 36% of cases carried *RET/PTC3* and none *RET/PTC1*.

Correlation with Tumor Behavior The correlation between *RET/PTC* and prognosis in human papillary carcinomas remains controversial. Several groups have suggested that *RET/PTC* is associated with more aggressive tumors, including those with locally advanced disease at presentation [47] and distant metastases [42]. Others reported quite the opposite findings, suggesting that papillary carcinomas harboring *RET/PTC* have a slow growth and do not progress to poorly differentiated or undifferentiated carcinomas [31,52,71]. However, these studies assume that all types of the rearrangement have comparable properties and considered them as a group.

It is conceivable, however, that different types of *RET/PTC* confer papillary carcinomas with distinct biological properties. Specifically, *RET/PTC1* may correlate with a favorable prognosis, whereas *RET/PTC3* with a more aggressive tumor behavior. This possibility is supported by several lines of evidence:

1. In a series of 17 papillary carcinomas from children and adolescents with no history of radiation exposure and an average follow-up of 5.4 yr, no local recurrence or distant metastases was observed in tumors with *RET/PTC1*, whereas those complications were seen in two out of three carcinomas with *RET/PTC3* [41,72].
2. *RET/PTC3* is associated with the tall cell variant and in some populations with the solid variant of papillary carcinoma, both of which are known to have a slightly more aggressive behavior, whereas *RET/PTC1* is more common in classic papillary carcinoma and in microcarcinoma.
3. The difference in the effects of *RET/PTC1* and *RET/PTC3* activation in vitro was observed in PC Cl3 rat thyroid cells transfected with both oncogenes [65]. The cells expressing *RET/PTC3* had a significantly higher proliferative activity, as determined by a fraction of cells in S and G_2/M phases of the cell cycle. Moreover, with the similar levels of gene expression and tyrosine phosphorylation, *RET/PTC3* transfected cells had an approximately threefold higher levels of mitogen-activated protein (MAP) kinase phosphorylation, demonstrating an increased signaling ability of *RET/PTC3* with respect to *RET/PTC1*.
4. In transgenic mice, thyroid-specific expression of *RET/PTC1* leads to the development of slowly progressing and virtually nonmetastatic thyroid cancers, which do not cause premature death of the animals [25,26,73]. In contrast, transgenic mice expressing *RET/PTC3* develop aggressive and metastatic thyroid tumors [27]. However, some caution should be used in direct comparison of these results because of the potential variability in the strains of mice, promoter constructs, and levels of chimeric gene expression between these animal models.

Although the association between the different types of *RET/PTC* and distinct tumor behavior has not been fully proven at this time, the evidence discussed in the preceding strongly suggests that it is likely to exist.

***TRK* REARRANGEMENT** Rearrangements involving another receptor tyrosine kinase gene, *TRK* (*NTRK1*, *TRKA*), have also been found in papillary thyroid carcinomas, but at a significantly lower rate. The *TRK* gene is located on chromosome 1q22 and encodes one of the receptors for the nerve growth factor [74,75]. It is expressed in neurons in both the peripheral and the central nervous system, and is involved in the regulation of growth,

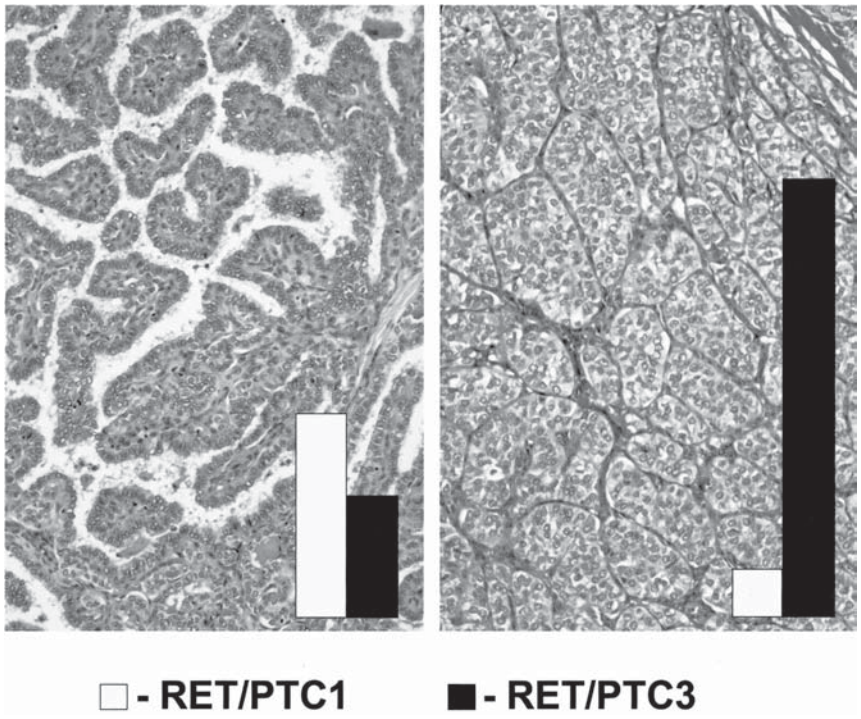

Figure 2 Correlation between classic papillary carcinoma (**left**) and *RET/PTC1* and between the solid variant of papillary carcinoma (**right**) and RET/PTC3 in post-Chernobyl tumors. (Based on the data reported in Nikiforov et al. [1997] [50].)

differentiation, and survival of these cells. In thyroid follicular cells, *TRK* can be activated through chromosomal rearrangement, which juxtaposes the intracellular tyrosine kinase domain of TRK to the 5' terminal sequence of different genes. To date, three fusion partner genes have been identified. Two of them, the tropomyosin (*TPM3*) gene [76,77] and the translocated promoter region (*TPR*) gene [78,79], are also on chromosome 1q, so that these fusions are formed by paracentric intrachromosomal inversion. Several variants of the *TPR–TRK* fusion (*TRK-T1, TRK-T2, TRK-T4*) exist due to variation of the breakpoint sites in both genes [75]. The third fusion partner, the *TFG* gene, is on chromosome 3 and its fusion with *TRK* is a consequence of the t(1;3) translocation [80]. Similar to the *RET* fusion partners in *RET/PTC* rearrangement, the genes fused with *TRK* are expressed in thyroid follicular cells and drive the expression and chronic ligand-independent activation of the tyrosine kinase domain of *TRK*. *TRK* rearrangement promotes neoplastic transformation of thyroid cells, as it has been demonstrated in transgenic mice with thyroid-specific expression of the *TPR–TRK* fusion gene [81]. The mice developed hyperplastic changes in their thyroid glands, and most animals older than 7 mo of age had papillary carcinomas.

TRK rearrangements have been reported in up to 10–15% of papillary thyroid carcinomas [47,75,82]. They can occur in tumors with classic papillary architecture and in different microscopic variants, including papillary microcarcinomas and the follicular and tall cell variants. All three types of the rearrangement are found with approximately similar frequency, and several tumors with still uncharacterized *TRK* fusion have been reported [47,82]. The association between TRK rearrangement and unfavorable prognosis has been suggested in a study of 119 papillary carcinomas followed on average for 5.7 yr [82]. It appeared that tumors carrying TRK rearrangement had a 60% local recurrence rate and a 27% tumor-related mortality, both values significantly higher that in tumors without the rearrangement.

***RAS* MUTATIONS** Point mutations of the *RAS* protooncogenes (N-*RAS*, K-*RAS*, and H-*RAS*) occur with variable frequency in all types of thyroid follicular cell–derived tumors. In papillary carcinomas, *RAS* mutations are relatively infrequent, and occur in 0–21% of tumors [83–88], with the exception of two observations reporting a much higher prevalence (54–67%) [89,90]. The presence of this genetic alteration, which is characteristic of follicular thyroid tumors, in a subset of papillary carcinomas raises the possibility that some tumors with papillary phenotype may be genetically related to follicular carcinomas and share with them certain biological properties. One of those would be the propensity for blood-borne metastases. Indeed, in one series of 91 papillary carcinomas followed on average for 14.1 yr, the incidence of *RAS* mutations was 28% in tumors associated with distant metastases and 8% in tumors confined to the thyroid [87]. In this study, the presence of the *RAS* mutation was also found to be an independent prognostic factor for aggressive behavior of papillary carcinoma. Biological properties and prevalence of *RAS* mutations in other types of thyroid tumors are discussed in greater detail later in the chapter.

***MET* OVEREXPRESSION** The *MET* gene is located on chromosome 7q31 and encodes a cell membrane tyrosine kinase receptor for the hepatocyte growth factor/scatter factor. Signaling through the *MET* receptor stimulates proliferation in differ-

Table 3
Prevalence of *RAS* Mutations in Thyroid Tumors

	Follicular adenoma	*Follicular carcinoma*	*Hürthle cell adenoma*	*Hürthle cell carcinoma*	*Papillary carcinoma*	*Poorly differentiated carcinoma*	*Anaplastic carcinoma*
Range	17–43%[a]	38–53%	0–4%	15–25%	0–21%[b]	18–27%	20–100%
Average in meta-analysis	34% (40/117)	45% (21/47)	3% (1/34)	16% (8/50)	15% (38/253)	24% (14/58)	55% (12/22)
References	[84,89,90,97,98]	[83,88–90,97,98]	[99,100]	[98–100]	[83–88]	[86,88,89]	[86,88,90,97,98]

[a]50% in a subset of microfollicular adenomas [97] and 85% in adenomas from iodine-deficient areas [102].
[b]Two additional studies reported an unusually high prevalence (54–67%) in papillary carcinomas [89,90].

ent cell types including thyroid, and results in a variety of other tissue-specific responses related to cell dissociation and motility (reviewed in [91]). In human cancers, abnormal *MET* signaling can result from activating point mutations within the gene, gene amplification, or through the creation of autocrine stimulatory loops [91]. *MET* overexpression has been found in 75–100% of papillary thyroid carcinomas [92–94]. However, no amplification or any structural alteration within the gene was identified in those tumors [92], suggesting that *MET* overexpression was due to transcriptional or posttranslational mechanisms. Indeed, it has been shown that activation of the *RET* and *RAS* genes in human thyroid cells leads to overexpression of *MET* [95]. This supports the possibility that *MET* disregulation in papillary carcinomas is likely to be secondary to mutations of other genes, rather than a primary genetic event.

FOLLICULAR THYROID TUMORS

A group of follicular thyroid tumors includes follicular carcinoma, Hürthle cell (oncocytic) carcinoma, and their benign counterparts—follicular adenoma and Hürthle cell (oncocytic) adenoma. All these neoplasms originate from thyroid follicular cells and characteristically grow forming variable-sized follicles. Hürthle cells differ from typical follicular cells in having a larger cell size and abundant granular eosinophilic cytoplasm, which has such appearance due to a massive accumulation of mitochondria. As a group, follicular carcinomas account for approx 15% of all thyroid malignancies, with about two thirds being a conventional type and one third a Hürthle-cell-type carcinomas [3]. It remains unclear whether Hürthle cell carcinomas and adenomas represent a subset of follicular tumors or are a separate type of thyroid neoplasm with a distinct genetic background and biological properties. Follicular carcinomas of a conventional type almost never involve regional lymph nodes, but in 10–20% of cases give distant metastases, most commonly to the lungs and bones. Hürthle cell carcinomas are known to spread via both regional lymph node and blood-borne metastases.

The preoperative diagnosis of follicular tumors is difficult because adenomas and carcinomas share similar cytolologic features, and the only two reliable criteria of carcinoma, capsular and vascular invasion, can be detected only on histologic evaluation of surgically removed specimens. In addition, some cellular hyperplastic nodules and follicular variants of papillary carcinoma may mimic follicular tumors in the fine needle aspiration (FNA) specimens. As a result, many follicular lesions are diagnosed as indeterminate or suspicious by FNA cytology and referred for surgery. However, only small fraction of those (8–17%) will prove to be malignant, so that the majority of patients are subjected to unnecessary surgery [96]. This demonstrates the importance of molecular markers that would improve the accuracy of preoperative diagnosis of follicular tumors.

RAS MUTATIONS

Oncogenic *RAS* Proteins The *RAS* genes encode highly related guanine nucleotide binding (G) proteins that are located at the inner surface of the cell membrane and play a central role in the transduction of signals arising from tyrosine kinase and G-protein-coupled receptors. In human cells, there are three potentially oncogenic *RAS* genes, H-*RAS*, K-*RAS*, and N-*RAS*, which encode homologous, but distinct 21-kDa proteins. In its inactive state, ras protein is bound to guanosine diphosphate (GDP). After activation, it releases GDP and binds guanosine triphosphate (GTP), initiating a cascade of MAP kinases. This pathway eventually leads to transcriptional activation of target genes, which direct the cell to enter the growth cycle. Normally, the activated ras protein quickly becomes inactive due to its intrinsic guanosine triphosphatase (GTPase) activity and the action of cytoplasmic GTPase-activating proteins, which catalyze the conversion of ras active GTP form to the inactive GDP form. In many human neoplasms, point mutations occur in the discrete domains of the *RAS* gene, which lead to either an increased affinity for GTP (mutations in codons 12 and 13) or inactivation of the autocatalytic GTPase function (mutations in codon 61). As a result, the mutant protein becomes permanently switched in the "on" position and constitutively actives its downstream signaling pathways.

Prevalence of *RAS* Mutations in Follicular Thyroid Tumors Activating point mutations of the *RAS* genes were among the first genetic alterations identified in thyroid tumors. With variable frequency, they occur in all types of neoplasms originating from thyroid follicular cells. Overall, their prevalence is higher in follicular tumors as compared to papillary carcinomas, and in follicular carcinomas as compared to adenomas (Table 3). Somatic missense mutation in codons 12/13 and 61 of one of the three *RAS* genes have been found in approx 45% of follicular carcinomas [83,88–90,97,98] and 35% of follicular adenomas [84,89,90,97,98]. In adenomas, the mutations appeared to be more common in tumors with a microfollicular growth pattern [97]. A lower incidence has been reported in Hürthle cell tumors,

in which only 0–4% of adenomas and 15–25% of carcinomas appeared to be affected [98–100]. However, whether or not *RAS* mutations are truly rare in Hürthle cell tumors remains unclear, as the total number of cases studied to date is quite low and in the largest series only N-*RAS* codon 61 mutations were searched for [99]. *RAS* mutations have also been detected in few cold adenomatous nodules and goiter nodules [84,98,101]. In most series, mutations involving N-*RAS* codon 61 and H-*RAS* codon 61 appear to be more common, although they have been found in different codons of all three genes, with no association between a particular *RAS* gene/codon mutation and tumor type or behavior.

In one observation, a significantly higher prevalence of *RAS* mutation was found in follicular adenomas and carcinomas from the areas of iodine deficiency as compared to iodine-sufficient regions [102]. Some early studies of thyroid tumors in experimental animals and humans also suggested the association between *RAS* (especially K-*RAS*) mutations and radiation exposure [103,104]. However, more recent analysis of large series of follicular and papillary tumors from patients subjected to therapeutic irradiation or accidental environmental irradiation failed to demonstrate such an association [105–107].

Consequences of *RAS* Activation in Thyroid Cells There is good evidence that *RAS* mutations are an early event in the progression of thyroid follicular tumors: (1) as discussed earlier in the chapter, they occur in all stages of tumorigenesis including benign follicular adenomas; (2) thyroid-specific expression of mutant K-*RAS* in transgenic mice leads to the development of benign thyroid nodules as well as follicular adenomas and rare carcinomas, the latter after additional goitrogen stimulation [108]; (3) *RAS* activation in thyroid cells in vitro results in the increased cell proliferation, but is not sufficient alone for complete transformation of thyroid cells. This was most clearly demonstrated in cultured normal human thyroid cells infected with retrovirus vector encoding the mutant H-*RAS* gene [109]. The introduction of H-*RAS* resulted in a dramatic stimulation of cell proliferation (from <1% to 36% cells in the S phase) and extension of their proliferative lifespan. Thus, while primary cultured human thyroid cells replicate slowly in vitro and senesce after two to five cell divisions, the H-*RAS* infected cells underwent up to 15–25 population doublings. During this period of rapid proliferation, they exhibited a partially transformed phenotype, but retained differentiated features including the expression of epithelial markers and production of thyroglobulin. Remarkably, after 15–25 population doublings, the cells stopped growing and underwent senescence. The termination of growth occurred despite undiminished expression of mutant *RAS* and was telomerase independent, but was associated with *de novo* expression of cyclin-dependent kinase inhibitor p16 [110]. This self-limited cell proliferation with the retention of differentiation properties recapitulates in many aspects the growth of human follicular adenomas, and is consistent with *RAS* being an initiating event in follicular tumor progression.

However, activating mutations of *RAS* also occur in many follicular and anaplastic thyroid carcinomas. This indicates that, besides the initial self-limited proliferation, these mutations may have some other effects and predispose cells to additional genetic alterations and transition to the overtly malignant phenotype. This may be due to the effect of *RAS* on stability of chromosomes. Indeed, in several cell types *RAS* has been shown to participate in controlling DNA synthesis and mitosis, particularly at the stage of assembly of mitotic spindle (reviewed in [111]). Acute activation of H-*RAS* in PCCL3 rat thyroid cells leads to chromosomal instability that manifests as centrosome amplification and chromosome misalignment in mitotic cells and as an increase in formation of micronuclei and small nuclear-like structures that form during mitosis as a result of chromosome missegregation [112]. However, it remains to be seen if these effects of *RAS* activation on chromosome stability, observed in animal cells and in the in vitro setting, also happen in human thyroid cells.

In human tumors, several studies have found a significant correlation between the presence of *RAS* mutations and metastatic behavior of follicular carcinomas, especially with respect to bone metastases [85,86,88]. However, because these mutations can also be seen in follicular adenomas, the mutation status alone cannot serve as an independent prognostic indicator and should be considered only in conjunction with the invasiveness of a follicular tumor determined by histologic examination.

PAX8–PPARγ REARRANGEMENT

Structure *PAX8–PPARγ* fusion has recently been identified in follicular thyroid tumors with cytogenetically detectable translocation t(2;3)(q13;p25) [113]. One involved gene, *PAX8*, is located on chromosome 2q13 and encodes a paired domain transcription factor that plays an important role in thyroid development and differentiation of follicular cells [114, 115]. Through its paired domain, PAX8 protein binds to the promoters of the thyroglobulin, thyroperoxidase, and sodium/iodide symporter genes and regulates their thyroid-specific expression [116,117]. Another partner of the rearrangement is the peroxisome proliferator-activated receptor *PPARγ*. PPARs are nuclear hormone receptors that belong to the steroid/thyroid/retinoid receptor superfamily and control a variety of genes involved in lipid metabolism [118]. Like other receptors of this family, they require heterodimerization with the retinoid X receptor (RXR) to bind to its DNA response elements (PPREs) to activate target gene expression. Three related *PPAR* genes have been identified: *PPARα*, *PPARβ*, and *PPARγ*. *PPARγ* is located on chromosome 3p25 and gives rise to three transcripts: *PPARγ1–3*, which share six common coding exons but differ at their 5'-ends as a consequence of the alternate splicing [118,119]. Both *PPARγ1* and *PPARγ2* are highly expressed in human adipose tissue and at low level in skeletal muscle, while *PPARγ1* expression has also been found in liver, heart, and some other tissues [118].

In follicular carcinomas with *PAX8–PPARγ* fusion, several transcripts are coexpressed, formed by the fusion of four *PAX8* variants (exons 1 to 7, 1 to 8, 1 to 9, or 1 to 7 plus 9) to *PPARγ* exons 1–6 [113] (Fig. 3). These different *PAX8* variants most likely result from the alternate splicing involving exons 8 and 9, as it occur in wild-type *PAX8* [120]. In our experience, the most commonly expressed *PAX8–PPARγ* transcripts in follicular thyroid carcinomas are those containing exons 1 to 9 and 1 to 7 plus 9 of *PAX8*. Irrespective of the specific *PAX8* splice variant, the fusion protein contains the paired and partial homeobox domains of PAX8 fused with the DNA binding, ligand binding, and RXR dimerization and transactivation domains of PPARγ.

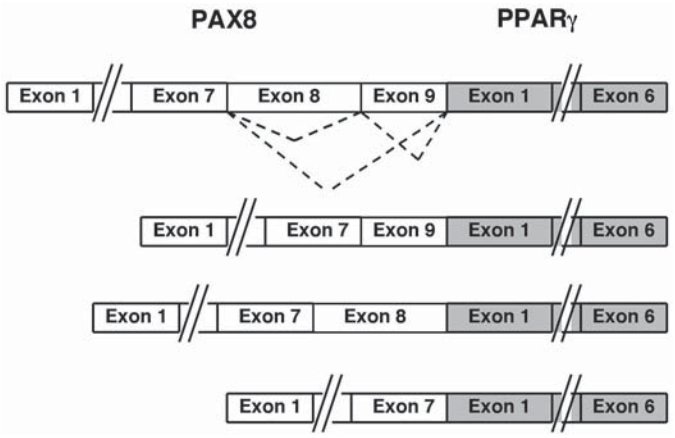

Figure 3 Structure of the various *PAX8–PPARγ* mRNA transcripts coexpressed in follicular thyroid tumors. They are likely formed by alternate splicing of the *PAX8* gene (*dashed lines*).

Table 4
Prevalence of *PAX8–PPARγ* Rearrangement in Thyroid Tumors

	Multinodular goiter	*Follicular adenoma*	*Follicular carcinoma*	*Hürthle cell tumors*	*Papillary carcinoma*	*Poorly differentiated and anaplastic carcinomas*
Kroll et al. [113]	0/10	0/20	5/8 (63%)		0/10	
Nikiforova et al. [121]	0/16	2/25 (8%)	8/15 (53%)	0/24	0/35	0/3

Prevalence in Thyroid Tumors To date, two studies have reported the prevalence of *PAX8–PPARγ* in thyroid tumors [113,121]. They demonstrated the occurrence of the fusion in 53–63% of follicular carcinomas, 0–8% of follicular adenomas, but in none of other benign and malignant thyroid lesions (Table 4). Follicular carcinomas tested positive and negative for *PAX8–PPARγ* did not differ significantly in patient's age, gender, thickness of capsule, tumor size, or growth pattern [121]. However, most follicular carcinomas with *PAX8–PPARγ* were overtly invasive, demonstrating multiple foci of tumor extension through the capsule and massive vascular invasion (Fig. 4). In contrast, the majority of tumors tested negative for the rearrangement were minimally invasive and demonstrated only single foci of capsular or vascular invasion [121]. This suggests that *PAX8–PPARγ* may confer follicular carcinomas with more aggressive growth and propensity for invasion. This possibility is also supported by a case report of an aggressive follicular carcinoma with translocation t(2;3)(q13;p25) detected cytogenetically in the primary tumor and its bone metastasis [122].

Among 16 follicular carcinomas reported in one study, the prevalence of *PAX8–PPARγ* was 42% (5/12) in tumors from the general population and 100% (3/3) in patients with a history of radiation exposure [121]. Although papillary carcinoma is by far the most common type of thyroid tumors associated with radiation exposure, an increased risk of follicular carcinomas has also been documented in these populations [123]. A higher prevalence of *PAX8–PPARγ* in this group suggests that radiation may promote the generation of *PAX8–PPARγ* rearrangement, similar to what has been shown for *RET/PTC* rearrangement in papillary carcinomas.

In addition to follicular carcinomas, *PAX8–PPARγ* was observed in two tumors meeting current diagnostic criteria for follicular adenoma [121]. Both of them had a trabecular growth pattern with minimal colloid, but showed no invasion. It is conceivable that they are follicular carcinomas that are biologically programmed for invasive growth but removed at a preinvasive stage. In support of this possibility, one of the two cases was a tumor apparently growing within but had not completely replaced a preexisting hyperplastic nodule. When further studied for the expression of galectin-3, a β-galactoside binding protein shown to be highly specific for malignant thyroid tumors [124], the tumor demonstrated a strong diffuse immunoreactivity with galectin-3 antibody, providing an additional evidence in favor of its malignant origin [196].

Interestingly, when a group of follicular carcinomas was studied for both *PAX8–PPARγ* rearrangement and *RAS* point mutations, it appeared that most of the tumors harbored either one or another genetic alteration, but not both. This suggests that follicular carcinomas may develop via two distinct molecular pathway initiated by either *PAX8–PPARγ* rearrangement or *RAS* mutation [196].

Possible Mechanisms of *PAX8–PPARγ*-Induced Transformation A high prevalence of *PAX8–PPARγ* rearrangements suggests an important role of this genetic event in the development of follicular thyroid cancer. However, the mechanisms of *PAX8–PPARγ*-induced transformation have not been clearly defined yet. Typically, chromosomal rearrangements promote carcinogenesis through the activation of an oncogene. This does not seem to be the case here. Thus, in U2OS cells transfected with *PAX8–PPARγ*, the fusion protein was found ineffective in

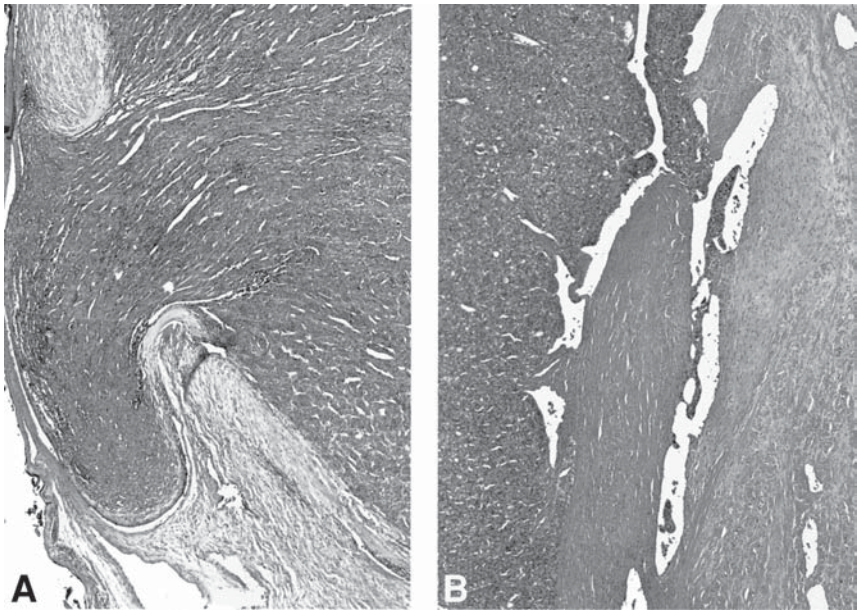

Figure 4 Follicular carcinomas carrying *PAX8–PPARγ* rearrangement are typically overtly invasive and demonstrated unequivocal capsular invasion (**A**) and multifocal vascular invasion (**B**).

inducing PPRE transactivation after stimulation with the activating agent troglitazone [113]. Moreover, when coexpressed in the same cell with wild-type *PPARγ*, the *PAX8–PPARγ* protein showed a dominant negative effect and completely abrogated wild-type *PPARγ* activity [113]. This suggests that deregulation of normal *PPARγ* function could serve as a mechanism of tumor progression. In this regard, it is of interest to note that *PPARγ* may indeed function as a tumor suppressor gene, and its activation can inhibit growth and promote differentiation of some cancer cell lines [118]. An important caveat is that in normal thyroid cells the wild-type *PPARγ* gene is only weakly expressed so that the protein is either undetectable or present in low amounts. This argues against the importance of *PPARγ* inactivation in thyroid cell transformation. Another possible mechanism of *PAX8–PPARγ* action would be through deregulation of *PAX8* pathways, as *PAX8* function is known to be critical for thyroid cell differentiation. Finally, it is conceivable that *PAX8–PPARγ* promotes tumorigenesis through another and still unknown mechanism.

LOSS OF HETEROZYGOSITY AND OTHER TYPES OF GENOMIC INSTABILITY

Loss of Heterozygosity Loss of heterozygosity (LOH) is characterized by physical deletion of large regions of chromosomes. Those regions that are consistently deleted in certain tumors have long been associated with the location of tumor suppressor genes whose loss of function is needed for tumor progression. At a late stage, however, most malignant neoplasms reveal a widespread loss and duplication of chromosomal regions or entire chromosomes, which reflect a general destabilization of the genome and have no association with specific genes residing in these areas.

Among thyroid tumors, follicular adenomas and carcinomas are characterized by a considerable rate of LOH, while papillary carcinomas have a stable genotype and low prevalence of allelic loss [125–127]. Indeed, a meta-analysis of the data reported in the literature revealed a 19.7% average rate of LOH per chromosome arm in follicular carcinomas, 5.8% in follicular adenomas, but only 2.5% in papillary carcinomas [125]. In addition, follicular carcinomas characteristically exhibit deletions involving multiple chromosomal regions (found in > 50% of tumors), while LOH for more than one loci is rare in follicular adenomas (4%), and virtually absent in papillary cancers [125]. The difference in LOH between various types of thyroid tumors has been best illustrated when the frequency of allelic loss throughout the entire genome, reported in multiple studies, was summarized for each tumor type (Fig. 5) [125].

In follicular carcinomas, the most commonly deleted regions found in different studies were on chromosomes 2p, 3p, 9q, 10q, 11p, 15q, and 17p, suggesting the presence of important tumor suppressor genes in these areas [125,126,128–131]. Some studies observed that 3p and 17q deletions were significantly higher in follicular carcinomas than in adenomas [128,132].

Hürthle cell neoplasms, when studied separately, show a comparable or even higher rate of LOH than conventional follicular tumors [129–131]. In Hürthle cell carcinomas, the most frequently lost regions were on chromosomes 3q and 18q in one study [129], and on chromosomes 1q, 2p, 8q, and 14q in another observation [131]. In the latter report, two markers (1q and 2p) showed a significantly higher rate of LOH in Hürthle cell carcinomas than in adenomas, with a 100% sensitivity and a 65% specificity in the detection of malignant tumors [131]. Hürthle cell adenomas and carcinomas are also characterized by frequent numerical chromosomal abnormalities, including both gains and losses of whole chromosome [100,133,134].

The pattern of LOH in various types of thyroid tumors correlates with those of DNA aneuploidy, which reflects a loss or gain of multiple whole chromosomes. Thus, fewer than 10% of papillary carcinomas are aneuploid [135,136], while 20–30%

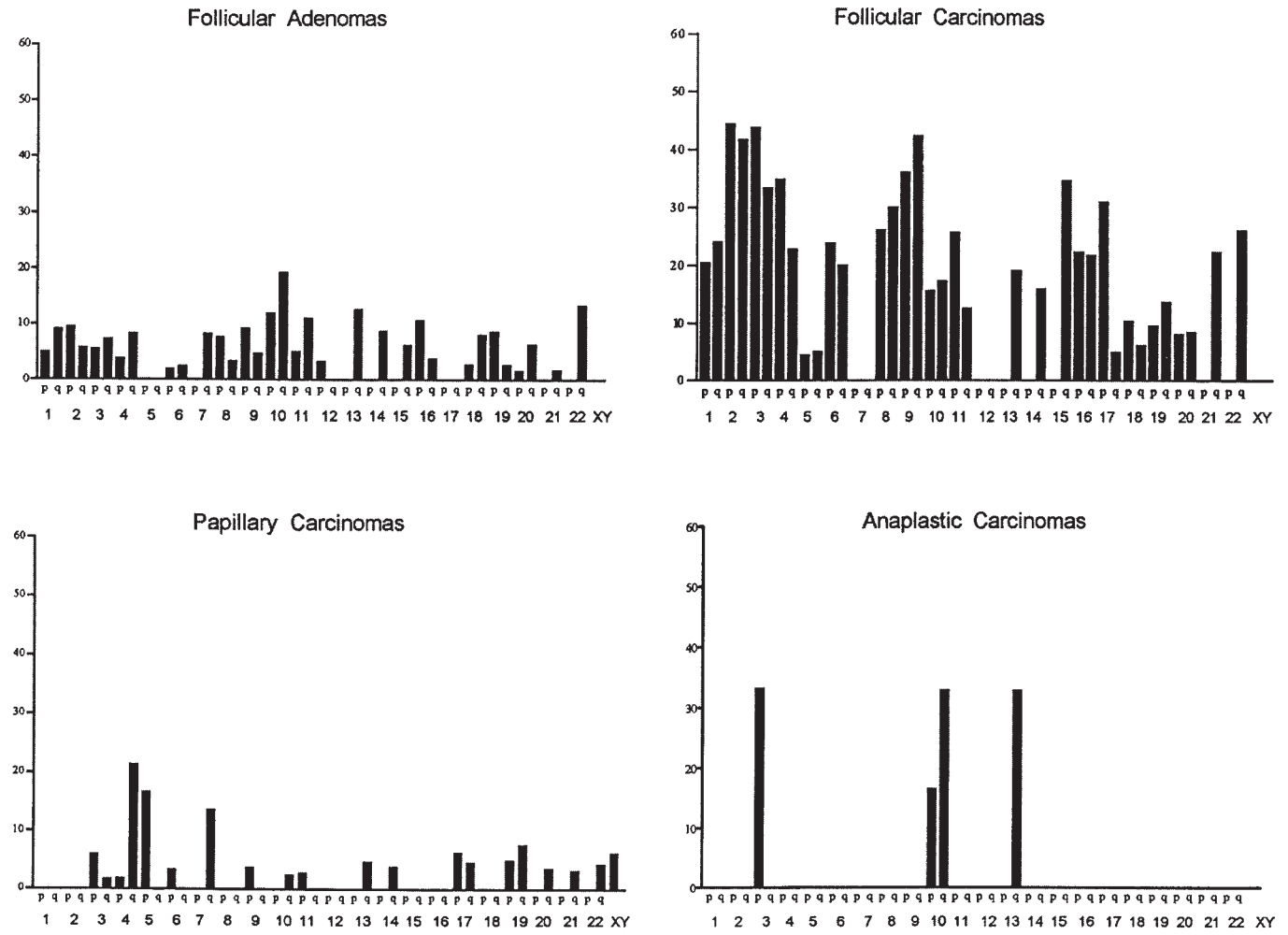

Figure 5 Meta-analysis of LOH per each chromosomal arm in various types of thyroid tumors. Note a sharp difference in the extent and frequency of LOH between papillary carcinoma and follicular tumors. Prevalence of LOH in anaplastic carcinoma shown here reflects only a few chromosomal arms studied at the time of the analysis, while more recent observations have found many other chromosomal regions frequently lost in anaplastic carcinomas. (Reproduced with permission from Ward LS et al. Studies of allelic loss in thyroid tumors reveal major differences in chromosomal instability between papillary and follicular carcinomas. J Clin Endocrinol Metab 83:525–530, 1998 [125]. Copyright, The Endocrine Society.)

of follicular adenomas and approx 50% of follicular carcinomas have aneuploid cell population [137–140]. The frequency of aneuploidy tends to increase from follicular adenomas to minimally to widely invasive follicular carcinomas, although a significant overlap between these groups exists, which precludes the usage of the test for diagnostic purposes. A sharp contrast in the prevalence of chromosomal instability, measured by LOH and DNA ploidy, between papillary carcinomas and follicular tumors highlights a fundamental difference in the molecular pathways involved in the development of these thyroid neoplasms.

Microsatellite and Minisatellite Instability Microsatellite instability is another marker of genomic instability, which manifests as an accumulation of mutations in simple tandem (di-, tri-, and tetranucleotide) DNA repeats and is secondary to loss-of-function of DNA mismatch repair enzymes. Microsatellite instability is common in certain inherited cancer syndromes (e.g., HNPCC) and some sporadic cancers. In thyroid tumors, it has been observed with a frequency of 14–33% in follicular carcinomas, and with lower prevalence in papillary carcinomas and Hürthle cell tumors [130,141–143]. Overall, microsatellite mutations are uncommon in thyroid tumors and rarely involve multiple foci, which argues against the presence of significant DNA replication error in these tumors.

Another type of genomic instability, minisatellite instability, manifests as an increased mutation rate in longer DNA repeats (in the range of 6–100 basepairs) [144]. The precise mechanism responsible for these mutations is unknown, but is most likely different from a defect in DNA mismatch repair. Somatic minisatellite instability has been found in 18% of pediatric radiation-induced papillary carcinomas, but not in sporadic tumors from adults [141]. To our knowledge, it has not been studied in follicular thyroid tumors or anaplastic carcinomas.

TSH-RECEPTOR AND G-PROTEIN MUTATIONS IN HYPERFUNCTIONING (TOXIC) THYROID NODULES Pituitary thyroid-stimulating hormone (TSH) is the major regulator of thyroid growth and function. Its cell membrane receptor, TSH-R, is a member of seven-transmembrane domain family of peptide

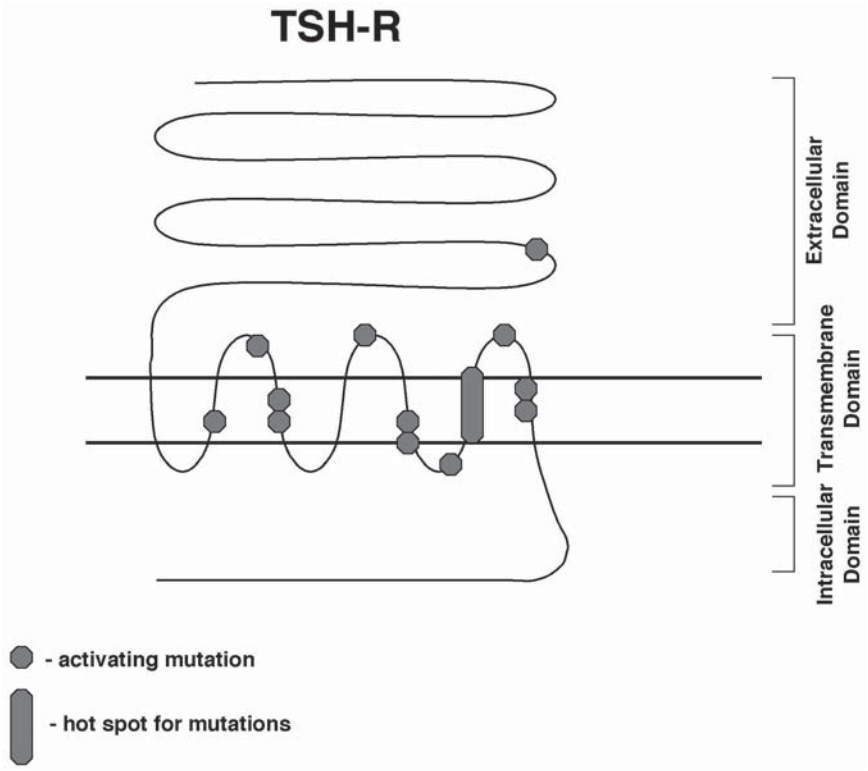

Figure 6 Schematic representation of the TSH receptor and location of activating point mutations in hyperfunctioning thyroid nodules.

receptors that are coupled with G proteins and signal through the cyclic AMP (cAMP) pathway. Upon TSH binding, the receptor is coupled to the α-subunit of the stimulatory G protein complex (GSα), which activates adenylate cyclase and generates the secondary messenger cAMP. Elevated levels of cAMP activate cAMP-dependent protein kinase A and, through a series of intermediate steps, lead to the stimulation of iodine uptake and metabolism, thyroid hormone synthesis and release, and other aspects of thyroid physiology. Thus, it can be predicted that constitutive activation of TSH-R or any other intermediates along the cAMP pathway will result in TSH-independent stimulation of thyroid cell function.

Indeed, activating point mutations in the *TSH-R* gene (located on chromosome 14q31 [145]) and *GSα* (*GSP*) gene (20q13 [146]) have been found in hyperfunctioning (toxic) nodules diagnosed as adenomas, adenomatous nodules (which, judged by their clonal origin, were probably true neoplasms [147]), nodules in multinodular goiter, and in occasional follicular carcinomas associated with hyperthyroidism. Most studies have reported a 48–82% rate of TSH-R mutations and a 3–6% rate of *GSα* mutations [148–150], although their prevalence varied markedly in some other series (reviewed in [151]). On the contrary, they are either totally absent or exceedingly rare in thyroid tumors with normal or decreased (cold nodules) radioiodine or ^{99m}Tc-pertechnetate uptake [152–154] . In both genes, point mutations cluster in the functionally important regions and lead to constitutive activation of the cAMP pathway. In *TSH-R*, they are located in the transmembrane domain, involved in interaction with the G-protein complex, and in the region of the extracellular domain responsible for the receptor affinity to TSH [155] (Fig. 6). In the *GSα* gene, mutations are limited to codons 201 and 227 and result in the inhibition of the α subunit intrinsic GTPase activity, leading to constitutive activation of the gene. It has been suggested that a higher rate of *TSH-R* mutations in hyperfunctioning thyroid nodules is due to the fact that it confers thyroid cells with more significant growth stimulation than *GSα* mutations, at least in the in vitro setting [156].

The role of these mutations in the development of a subset of hyperfunctioning thyroid tumors has been confirmed in transgenic mice with thyroid-specific expression of the mutant *GSα* gene [157]. Indeed, virtually all animals older than 8 mo of age developed thyroid nodules composed of cells demonstrating an elevated cAMP level and high uptake of radioiodine.

PTEN *PTEN* (*MMAC1* or *TEP1*) is a tumor suppressor gene, which is located on chromosome 10q23 and encodes a dual-specificity phosphatase with the ability to dephosphorylate both lipid and protein substrates. As a major lipid 3-phosphatase, it serves as a negative regulator of the phosphoinositol-3-kinase (PI3K)/AKT pathway, which is critical for cell survival, proliferation, and apoptosis. It is also able to dephosphorylate protein substrates on serine, threonine, and tyrosine residues and participates in the regulation of another set of signaling pathways, including the MAPK pathway. *PTEN* is the gene responsible for Cowden's syndrome, an autosomal dominant disorder characterized by multiple hamartomas affecting derivatives of all three germ layers and by an increased risk of breast, thyroid, and endometrial tumors. Thyroid abnormalities are found in 50–67% of the affected individuals and include multinodular

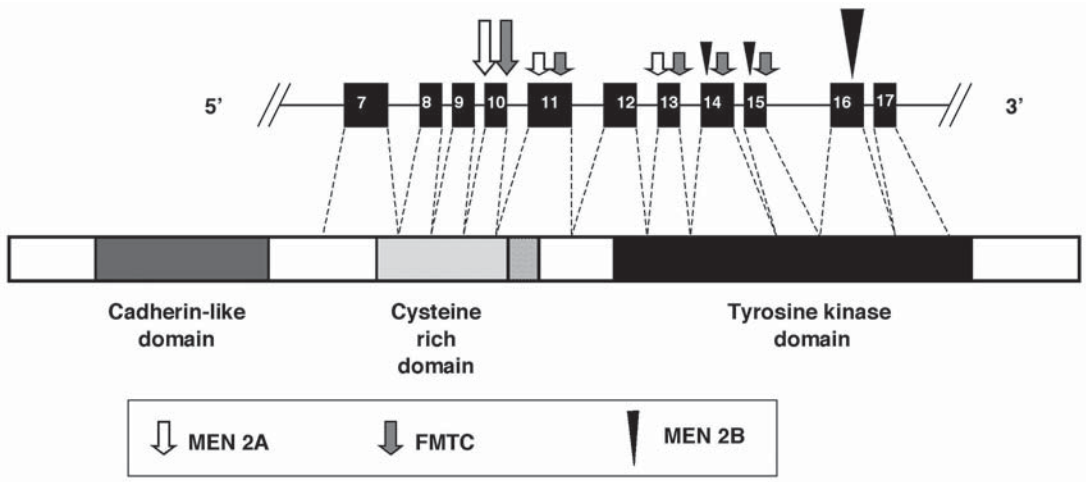

Figure 7 Distribution of germline point mutations in the *RET* gene and protein in patients with *MEN2A*, *MEN2B*, and *FMTC*. Exons of the gene are represented by *boxes* and introns by connecting *horizontal lines*. *Larger symbols* correspond to a hot spot for mutations in each syndrome.

goiter, follicular adenomas, and carcinomas (typically follicular carcinomas) (reviewed in [158]). Germ-line mutations located in exon 5 and other exons of the *PTEN* gene are found in more than 80% of Cowden's syndrome patients, making *PTEN* an obvious candidate gene for sporadic thyroid tumors, especially follicular adenomas and carcinomas.

Somatic point mutations of *PTEN*, however, are rare in sporadic thyroid tumors, being reported only in single cases of follicular adenoma and follicular, papillary, and anaplastic carcinoma [159–161]. At the same time, many thyroid tumors have a decreased level of the *PTEN* mRNA and protein, suggesting that inactivation of this tumor suppressor gene can occur through the mechanisms other than point mutation [159,162]. One of the potential mechanisms is LOH. Indeed, the region on 10q22–23 has been found to be deleted in up to 27% of follicular adenomas and carcinomas [160,161,163]. However, this finding cannot establish firmly the role of PTEN inactivation in thyroid carcinogenesis, as other important genes may reside in this chromosomal region. One of those could be the *MINPP1* gene, which was mapped to chromosome 10q23.3 and recently found to have a somatic mutation in one follicular carcinoma [164]. Another possible mechanism of *PTEN* inhibition can be proposed on the basis of a recent finding that in some cell types the expression of *PTEN* is regulated by *PPARγ* [165]. Therefore, it would be conceivable that in thyroid follicular carcinomas carrying *PAX8–PPARγ* rearrangement, which has a dominant negative effect on wild-type *PPARγ*, *PTEN* was downregulated secondary to the inhibition of the *PPARγ* pathway. However, our preliminary study of 18 follicular carcinomas and adenomas showed no significant correlation between the presence of *PAX8–PPARγ* rearrangement and *PTEN* expression detected by immunohistochemical analysis (Nikiforov et al., unpublished).

MEDULLARY THYROID CARCINOMA

Medullary carcinoma originates from calcitonin-producing parafollicular, or C cells and constitutes approx 3.5% of all thyroid cancers [1,3]. Although most of the tumors are sporadic (nonfamilial), approx 20% occur in the setting of multiple endocrine neoplasia (MEN) 2A or 2B syndromes or as familial medullary thyroid carcinoma (FMTC), all inherited as autosomal dominant traits. In addition to medullary carcinoma, MEN 2A is also characterized by pheochromocytoma and parathyroid hyperplasia or adenoma, and MEN 2B by pheochromocytoma, neuromas, or ganglioneuromas of skin and mucosal membranes, and skeletal abnormalities. In contrast to sporadic medullary carcinomas, those associated with familial syndromes typically occur in younger patients, even during childhood, and are often multicentric and accompanied by C-cell hyperplasia [166,167].

***RET* POINT MUTATIONS** Over the last decade, the *RET* protooncogene has been identified as the key molecule associated with the development of medullary carcinoma, including both familial and sporadic forms of the disease. In this setting, *RET* is activated by point mutation, in contrast to its activation via chromosomal rearrangement in papillary thyroid carcinomas. Germline missense mutations in specific functional regions of the gene are found in almost all patients with familial forms of medullary carcinoma (Fig. 7). In *MEN 2A* and *FMTC*, they are typically located within the cysteine-rich region in the extracellular domain [168,169]. However, while almost 90% of *MEN 2A* mutations affect a single codon 634, in *FMTC* they are more evenly distributed along the cysteine rich region of the gene (reviewed in [170]). In *MEN 2B*, more than 90% of mutations involve codon 918 in the intracellular tyrosine kinase domain [171,172]. These germline mutations lead to *RET* activation and conversion into an oncogene through different mechanisms [173–176]. *MEN 2A* and *FTMC* mutations replace cysteine with another amino acids, which results in ligand-independent dimerization of *RET* and constitutive activation of the receptor. As for the codon 918 mutation in *MEN 2B*, it alters substrate specificity of the *RET* tyrosine kinase, leading to phosphorylation of new intracellular targets. The tumorigenic role of mutant *RET* has been confirmed in transgenic mice with C-cell-targeted expression of *RET* mutated at codon 634 [177]. Almost all animals developed bilateral C-cell hyperplasia at as

early as 3 wk of age, and subsequently present with multicentric medullary carcinomas.

The discovery of the gene responsible for the familial forms of medullary carcinoma and the availability of the reliable tests for *RET* mutations have changed dramatically the management and prognosis for these patients. Thus, early genetic screening of family members for *RET* mutations is now the standard of care, and prophylactic thyroidectomy for the affected individuals is commonly used to prevent the development of medullary carcinoma, which is the most lethal component of these inherited syndromes [178]. Preventive surgery administered on the basis of molecular testing results in the removal of thyroid tumors at the early stage, so that most surgical specimens from these patients reveal either medullary carcinomas of <1 cm in size or just a premalignant diffuse C-cell hyperplasia [179].

In sporadic medullary carcinomas, somatic *RET* mutations are found in 23–70% of cases (reviewed in [180]). The vast majority of those affect codon 918, although they have also been identified in few other regions and the number of novel mutation spots continues to grow [181].

POORLY DIFFERECTIATED CARCINOMA AND ANAPLASTIC CARCINOMA

Poorly differentiated carcinoma is a rare thyroid tumor that arises from follicular cells and is characterized by a less favorable prognosis in comparison with well-differentiated papillary or follicular carcinomas. Anaplastic (undifferentiated) carcinoma represents the most undifferentiated type of thyroid tumors and is one of the most aggressive human neoplasms. It constitutes <2% of all thyroid malignancies [3]. In thyroidectomy specimens, both poorly differentiated and anaplastic carcinoma may coincide with the foci of well differentiated papillary, follicular, or Hürthle cell carcinoma. This indicates that many of them arise from the preexisting well-differentiated tumors following a stepwise progression: well-differentiated carcinoma derived from follicular cells → poorly differentiated carcinoma → anaplastic carcinoma.

***RAS* MUTATIONS** As discussed above, point mutations of the *RAS* genes occur in all types of follicular cell–derived thyroid tumors. They have been reported in 18–27% of poorly differentiated carcinomas and in approx 60% of anaplastic carcinomas (Table 3). The fact that they occur in benign adenomas and abundant experimental data suggest that *RAS* mutations confer thyroid cells with a limited growth potential and are not sufficient alone to promote the development of a highly malignant anaplastic carcinoma. It is more likely that mutant *RAS* predisposes cells to the accumulation of additional genetic abnormalities, possibly by promoting chromosome instability or other point mutations, such as those of the *p53* gene. This can be illustrated by a case report of an anaplastic carcinoma developed in a well-differentiated follicular carcinoma, where the *RAS* mutation was found in both tumor components, whereas *p53* was found only in the anaplastic carcinoma [182].

***p53* MUTATIONS** Point mutations of the *p53* tumor suppressor gene are among the most common mutations found in human cancer. *p53* is located on chromosome 17p13 and encodes a nuclear transcription factor that plays a central role in the regulation of cell cycle, DNA repair, and apoptosis. It exerts these functions largely by its ability to transactivate expression of genes coding for proteins such as p21/WAF1 that induce G_1 arrest by inhibiting cyclin-dependent kinase complexes. *p53* becomes overexpressed immediately after the exposure to DNA-damaging agents, such as ionizing radiation and certain chemotherapeutic drugs, and causes transient cell cycle arrest, presumably to allow DNA repair to proceed under more favorable conditions. However, if the damage is severe, it initiates apoptosis to prevent perpetuation of the flawed cell. Alteration of *p53* function in cancer cells by inactivating point mutation or by deletion is believed to result in progressive genome destabilization, rapid accumulation of additional mutations, and evolution of more malignant clones.

In thyroid tumors, point mutations of *p53* are a late event, being reported in 67–83% of anaplastic carcinomas and 17–38% of poorly differentiated carcinomas, but only in single cases of follicular and papillary carcinomas [183–187]. Most of them involve exons 5–8 of the gene and alter its DNA binding properties. It appears that *p53* inactivation in thyroid cells is not only responsible for accelerated tumor growth, but is also associated with the progressive loss of differentiated markers. Indeed, the recovery of wild-type *p53* expression in cultured thyroid anaplastic carcinoma cells leads to the reduction in proliferation rate, reexpression of thyroid specific genes (*TPO*, *PAX-8*, and others), and reacquisition the ability to respond to TSH stimulation [188,189]. This suggests that the progressive loss of differentiation in poorly differentiated and anaplastic carcinomas is mediated, at least in part, by the inactivation of the *p53* gene.

β-CATENIN β-Catenin is a cytoplasmic protein, encoded by the *CTNNB1* gene on chromosome 3p22–3p21.3 [190,191], that plays an important role in E-cadherin-mediated cell–cell adhesion and is also an important intermediate in the wingless (Wnt) signaling pathway. Normally, in the absence of Wnt signaling, the protein is located at the inner surface of cell membrane and at a low level in the cytoplasm, where it is rapidly degraded by the adenomatous polyposis coli (APC) multiprotein complex. Wnt binding stabilizes the protein, which accumulates in the cytoplasm and translocates to the nucleus, where it upregulates the transcriptional activity of cyclin D1, c-*myc*, c-*jun*, and other genes. Point mutations in the phosphorylation sites of β-catenin (coded on exon 3 of the gene) stabilize the protein by making it insensitive for APC-induced degradation. This results in the accumulation of β-catenin in the nucleus and constitutive activation of target gene expression. Point mutations in exon 3 of the gene and/or aberrant nuclear accumulation of β-catenin have been found in various human neoplasms and are believed to be important in carcinogenesis (reviewed in [192]).

In thyroid tumors, point mutations in exon 3 of *CTNNB1* have been reported in 25% of poorly differentiated carcinomas and 66% of anaplastic carcinomas, but not in well-differentiated carcinomas [193,194]. Most of the tumors carrying the mutation also demonstrated an aberrant nuclear expression of the protein determined by immunohistochemical analysis, although there was no full correlation between these findings. Because the Wnt signaling pathway is functionally active in human thyroid cells [195], its constitutive activation by mutated β-catenin is likely to play a role in the progression to poorly differentiated and anaplastic carcinoma.

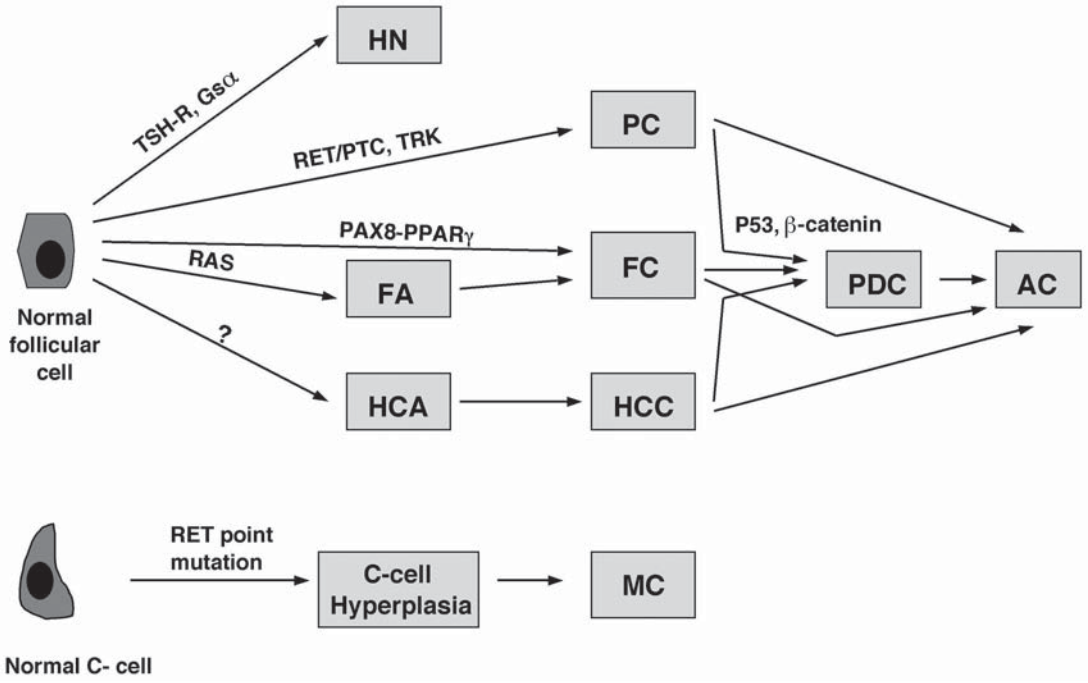

Figure 8 Putative scheme of thyroid tumorigenesis and molecular events in hyperfunctioning nodules (HN), papillary carcinoma (PC), follicular adenoma (FA), follicular carcinoma (FC), Hürthle cell adenoma (HCA), Hürthle cell carcinoma (HCC), poorly differentiated carcinoma (PDC), anaplastic carcinoma (AC), and medullary carcinoma (MC).

SUMMARY

Genetic events in various types of thyroid tumors are summarized in the hypothetical scheme of thyroid tumor development and progression on Fig. 8.

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11 Adrenal Cortex

Hironobu Sasano, MD, PhD,
Takashi Suzuki, MD, PhD, and Takuya Moriya, MD, PhD

HISTORICAL OVERVIEW

The adrenal gland was described in detail nearly 400 yr ago, but it was Thomas Addison who provided the first concrete evidence of the vital nature of the adrenal glands through an accurate description of the syndrome of adrenocortical insufficiency, termed Addison's disease, in 1855. Since then, numerous attempts have been made to characterize the biological or biochemical nature of substances produced and secreted by the adrenal glands mainly through the analysis of an extract from adrenals. Kendall at the Mayo Clinic prepared crystalline cortical extracts in 1934, which led to the precise characterization of adrenocortical hormones and their routine use in clinical practice. The successful use of cortisone in treatment of rheumatoid arthritis in 1949 by Hench and Kendall at the Mayo Clinic further enhanced interest in the clinical application of corticosteroids. In contrast to these dramatic improvements and successful use of adrenal corticosteroids, the histopathology of adrenocortical diseases had not necessarily kept pace with these advancements of clinical and/or basic endocrinology. The introduction of electron microscopy demonstrated the presence of relatively specific organelles involved in steroidogenesis in adrenocortical parenchymal cells, including mitochondria with specific cristae and smooth endoplasmic reticulum. However, these findings could not demonstrate the functional localization of corticosteroids, that is, which cell types produce what types of corticosteroids, one of the most important aspects of adrenocortical pathology. Introduction of immunohistochemistry yielded remarkable improvements in endocrine pathology in general. Antibodies against corticosteroids that were developed mainly for radioimmunoassay or enzyme-linked immunoassay have been employed in immunohistochemical evaluation of adrenocortical disorders. However, it was difficult to fix steroids in the tissue and steroids were easily extracted into organic solvents employed in the process of immunostaining. In addition, immunoreactivity recognized by antibodies against corticosteroids may represent steroids stored, synthesized, or bound to carrier proteins. Therefore, immunostaining of steroids themselves could not provide any biologically relevant information as to functional pathology.

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Corticosteroidogenesis is in general catalyzed by specific enzymes, mainly cytochrome P450. Immunohistochemistry of these enzymes specifically involved in the steps of corticosteroidogenesis demonstrated for the first time an endocrine–pathological correlation of the human adrenal cortex and its disorders [1–9]. In this chapter, the normal adrenal cortex is briefly described, followed by a description of the histopathological features of major adrenocortical diseases with emphasis on differential diagnosis and application of immunohistochemical and/or molecular techniques.

THE NORMAL ADRENAL CORTEX

HISTOLOGY Adrenocortical steroidogenesis is well known to be under the control of the hypothalamo–pituitary–adrenal axis. It is also important to note that the histology of the "normal" human adrenal cortex also changes according to the status of hypothalamo–pituitary–adrenal axis. In the adrenal of normal subjects, three major zones—the zona glomerulosa, zona fasciculate, and zona reticularis—can be appreciated by their histologic structure and their cytologic features [10]. The zona glomerulosa is situated around the periphery of the cortex beneath the capsule and forms rounded nests or clusters of cortical cells [11,12]. The zona glomerulosa cells are in general small, mitochondria are few in number, and smooth endoplasmic reticulum is small and relatively not well developed. The zona fasciculata is composed of the clear cortical cells arranged in cords or columnlike fashion. The zona reticularis occupies the inner one third to one quarter of the cortex. Cortical cells of the zona reticularis have eosinophilic compact cytoplasm and are arranged in anastomosing cords [11,12]. These morphological features of the "normal" or "nonpathological" human adrenal cortex are generally observed in the adrenals of subjects who are not under chronic stress including autopsy specimens from individuals who died suddenly or a great majority of surgical pathology specimens from adrenalectomy concomitant with nephrectomy for renal cell carcinoma. It is very important for pathologists to recognize the following two frequent morphological features of the adrenals frequently observed in otherwise "nonpathologic" adrenals.

Lipid Depletion Adrenocorticotrophic hormone (ACTH) is known to secrete from the anterior pituitary gland in response to stress and cause the adrenal cortex to produce and secrete

cortisol. Increased circulating levels of ACTH caused by the stress generally resulted in stimulation of the adrenocortical cells and subsequent lipid depletion of the fasciculata cells following utilization of intracellular cholesterol stores [1,10, 13]. The extent of these morphological changes in the human adrenal depends on the length and the severity of the stress. The ratio of compact cortical cells with lipid depletion in the zona fasciculata can be focal to complete. The expression of 3β-hydroxysteroid dehydrogenase (3βHSD) was demonstrated throughout the adrenal cortex with lipid depletion in the great majority of autopsy adrenal specimens with long-term disorders, in contrast to the adrenal cortex obtained from nephrectomies described earlier which demonstrated immunoreactivity of the enzyme, mostly in the outer zona fasciculate [1]. Therefore, postmortem examination of the adrenal cortex may provide insights into the extent of individual response to antemortem stress.

Cortical Nodules When carefully examining adult adrenal specimens obtained from autopsy or surgery, some degrees of nodularity of the adrenal cortex can be seen in almost all cases. The frequency of these adrenocortical nodules increases with age of the patients, and with hypertension and/or diabetes mellitus [13]. These cortical nodules can be composed of clear cortical cells or compact cortical cells with or without pigments. Adrenal glands with cortical nodules are much more frequently associated with various degrees of atherosclerotic or hypertensive changes of the arteries. In addition, the size or the number of cortical nodules is generally associated with the antermortem clinical severity of hypertension and/or hyperlipidemia and/or diabetes mellitus of the patients. Based on these findings we postulate that adrenocortical nodules occurred as a result of localized compensatory overgrowth of adrenocortical cells in response to altered intraadrenal blood flow or the localized ischemic changes of the cortical cells adjacent to the cortical nodules as shown in Fig. 1. Adrenocortical nodules by no means represent neoplastic or preneoplastic changes of the adrenal cortex, which is very important for pathologists, clinicians, and radiologists to recognize when managing patients with incidental adrenocortical mass lesion(s), described later in this chapter. In addition, it is also important to describe these changes when evaluating autopsy adrenal specimens.

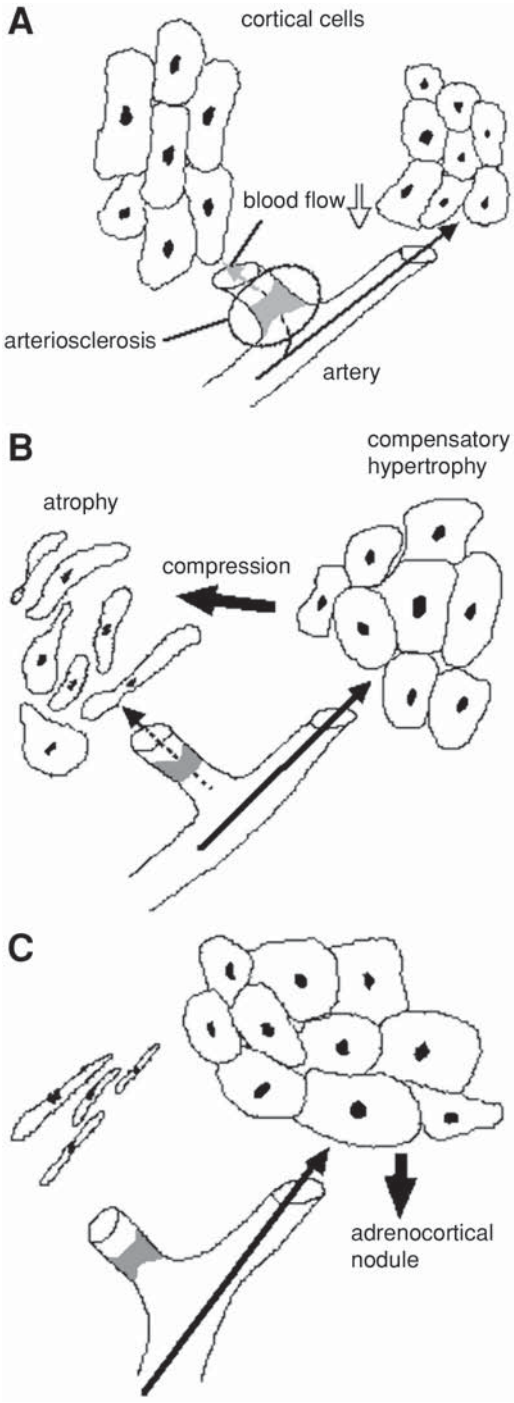

Figure 1 Schematic illustrations of the possible sequence of adrenocortical nodule development. Localized ischemic changes due to atherosclerosis or other causes are considered to result in ischemia and degeneration in the cortical areas supplied by these intraadrenal vessels. In response to these changes, the adrenal cortex adjacent to these areas proliferates in a compensatory manner.

HYPERCORTISOLISM

The etiology of hypercortisolism can be classified into ACTH-dependent and ACTH-independent. Surgical pathologists rarely receive adrenal specimens from ACTH-dependent hypercortisolism owing to the recent development and widespread use of transpheroidal surgery of pituitary ACTH-producing adenomas and other effective conservative medical and/or radiological treatments. Bilateral adrenalectomy is rarely performed in the great majority of institutions and hospitals, although it is true that only bilateral adrenalectomy can alleviate the symptoms of ACTH-dependent hypercortisolism despite the potential of developing Nelson's syndrome in some patients. However, even in these cases, surgical pathologists rarely experience diagnostic difficulties with bilateral adrenalectomy specimens if sufficient clinical and hormonal findings are provided by clinicians. In addition, there have been no established cases that reported that ACTH-dependent hypercortisolism resulted in histologically confirmed functioning primary adrenocortical neoplasms. Therefore, in this chapter, we focus on ACTH-independent hypercortisolism.

ACTH-independent hypercortisolism or Cushing's syndrome can also be subclassified into neoplastic and non-neoplastic adrenocortical lesions [13]. The great majority of non-neoplastic

ACTH-independent hypercortisolism is bilateral but that of neoplastic ACTH-independent hypercortisolism is unilateral. If resected adrenal glands demonstrate the features of non-neoplastic ACTH-independent hypercortisolism, it is very important to explore the contralateral adrenal gland to examine for the presence of the disease. It has thus become very important for surgical pathologists to differentiate neoplastic from non-neoplastic lesions in evaluation of resected adrenals from patients with ACTH-independent hypercortisolism, although the majority of these patients had neoplastic lesions.

In addition, there have been an increasing number of case reports of asymptomatic cortisol-producing neoplasms that secrete cortisol without clinical evidence of Cushing's syndrome. These cases have been designated as "pre-Cushing's syndrome" or "preclinical Cushing's syndrome" [14–16], although there are no reported cases in which the transition from "pre-Cushing's syndrome" to "full-blown Cushing's syndrome" and thus there have been controversies as to the term "pre-Cushing's" or "preclinical." Some cases of these adrenal incidentaloma or "pre-Cushing's adenoma" were reported to cause clinical adrenocortical insufficiency after removal [16,17]. Therefore, it has become very important to manage these patients with adrenal incidentalomas, especially with regard to whether the adrenocortical lesions should be resected or not, and if they are scheduled to be removed, it is also important to detect preoperatively subtle hyperfunctioning adrenocortical neoplasms, especially associated with subtle hypercortisolism.

In this chapter, differential diagnosis between ACTH-independent neoplastic and non-neoplastic hypercortisolism and between hormonally or clinically active and inactive Cushing's adenoma is described.

NEOPLASTIC ACTH-INDEPENDENT HYPERCORTISOLISM Among the surgical pathology specimens of ACTH-indepen-dent hypercortisolism, the great majority (>90%) of the lesions are neoplastic in all institutions and hospitals. When receiving these specimens in the diagnostic pathology laboratory, it is very important to study whether neoplastic ACTH-independent hypercorticolism is caused by adenomas or carcinomas.

Adrenocortical adenoma associated with Cushing's syndrome is also termed Cushing's adenoma. Grossly, in our experience the great majority of Cushing's adenoma demonstrated small and well-circumscribed neoplasms with tan to light brown color on the cut surface. In our series, the majority of tumors demonstrated a heterogeneous appearance in color on the cut surface. A tumor that is golden yellow on the cut surface, as observed in aldosteronoma, is infrequent but does exist. The tumor may or may not be encapsulated. In our series, the ratio of clear and compact adrenocortical tumor cells determined the color of the cut surface of these neoplasms. A tumor in which the ratio of clear cells is high generally demonstrated yellow to light tan color on the cut surface and vice versa. Foci of hemorrhage and necrosis are rarely observed. If present, the possibility of adrenocortical carcinoma should be suspected. The cut surface of a small number of the cases presents a homogeneous dark brown and black appearance, and these adrenocortical tumors are designated as black adenoma (Fig. 2). Microscopically, almost all the cortical tumor cells of black adenoma are composed of eosinophilic lipid-sparse compact cytoplasm with many

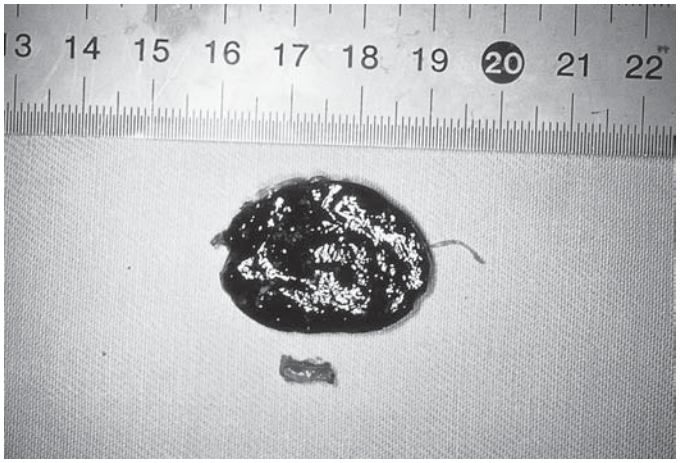

Figure 2 Macroscopic features of black adenoma associated with Cushing's syndrome. The cut surface of the tumor demonstrated relatively homogeneous dark brown to black appearance. Light microscopic examination generally revealed tumor cells with abundant eosinophilic cytoplasm and lipofuscin granules. (Color illustration appears in insert following p. 148.)

lipofuschin granules. There are no significant differences of serum steroid profiles or secretion patterns of corticosteroids between black and nonblack adenomas and between light tan and brown Cushing's adenomas [10]. Compact tumor cells generally exhibited much more prominent immunoreactivity of $P450_{c17}$ [6] and $P450_{c11}$ [7], which are involved in the final pathways of cortisol production. However, the ratio of compact tumor cells does not appear to be correlated with overall cortical output from the tumor tissue [10].

One of the characteristic morphological features of Cushing's adenoma is the marked atrophy of attached non-neoplastic adrenals (Fig. 3). The non-neoplastic adrenal may occasionally appear nonatrophic due to tangential sectioning, but even in these cases microscopically cortical cells demonstrated the presence of cellular cortical atrophy, including the clear cytoplasm with abundant lipid and the absence of compact cells (Fig. 3). The expression of steroidogenic enzymes, especially that of dehydroepiandrosterone sulfotransferase (DHEA-ST) is markedly diminished in these non-neoplastic attached adrenal cortices [18]. These features all reflect suppression of the hypothalamo–pituitary–adrenal axis due to neoplastic autonomous production of corticosteroids. Therefore, it is helpful for clinicians if pathologists describe the presence of cortical atrophy of the non-neoplastic attached adrenal, that is, pathological findings consistent with ACTH-independent hypercortisolism in the surgical pathology report of patients with Cushing's adenoma.

NON-NEOPLASTIC ACTH-INDEPENDENT HYPERCORTISOLISM Cases of non-neoplastic ACTH-independent hypercortisolism are rare, as described above but include interesting disorders. Much progress has been made, especially in molecular and cellular aspects of primary pigmented nodular adrenocortical disease (PPNAD) and ACTH-independent adrenocortical macronodular hyperplasia (AIMAH), which are rare but established and important causes of Cushing's syndrome.

Primary Pigmented Nodular Adrenocortical Disease PPNAD is an unusual cause of Cushing's syndrome first described

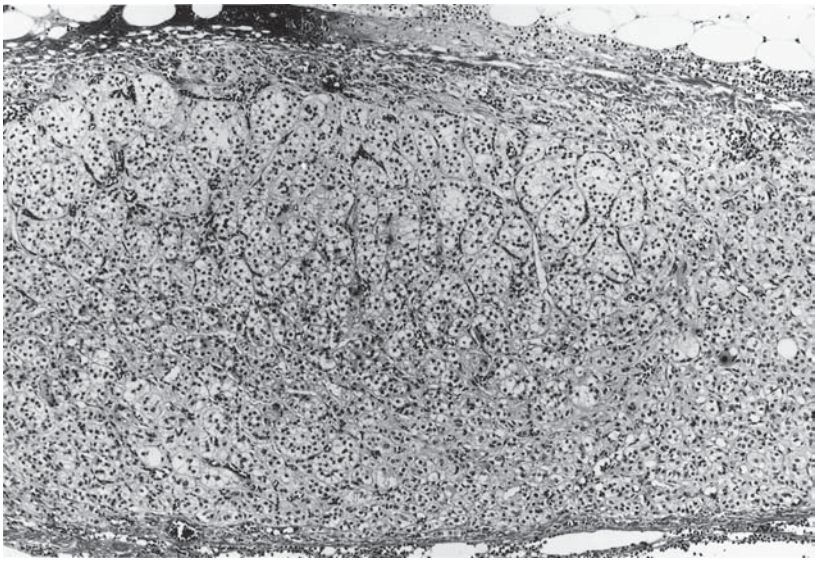

Figure 3 Light microscopic features of attached adrenal in Cushing's adenoma. Both architectural, that is, thin cortex, and cytological cortical atrophy, that is, marked clear cytoplasm, were detected.

by Chute et al. in 1949 [19]. PPNAD is clinically characterized by an ACTH-independent bilateral adrenocortical hyperfunction without demonstrable autonomous functioning neoplasms [20–22]. Adrenals of PPNAD are not enlarged and may appear normal in size by computerized tomography (CT) scan or other diagnostic radiological techniques. In a number of instances, PPNAD has a familial predirection with involvement of more than one family member. In addition, PPNAD can occur in association with unusual diseases including large cell calcifying Sertoli cell tumor, cardiac myxoma, cutaneous myxoma, myxoid mammary fibroadenomas, and spotty cutaneous pigmentation [1,12,13]. The combination of these disorders with PPNAD has been called "Carney's complex," named after Dr. Carney at the Mayo Clinic. Among these unusual concomitant diseases, it is very important to remember that cardiac myoma can be lethal in some patients and echocardiography needs to be done in patients with PPNAD. PPNAD due to the Carney complex is transmitted as an autosomal dominant trait but more than one gene may be involved in this unigue disorder. Grossly, in PPNAD the adrenals are not enlarged, weighing 2.6–9.6 g in our series [22]. Multiple tan to black nodules were scattered throughout the cortex on the cut surface of the adrenals of patients with PPNAD. Microscopically, these nodules were well circumscribed but not encapsulated and were composed predominantly of compact cortical cells containing abundant eosinophilic cytoplasm and little lipid (Fig. 4). Immunoreactivity and mRNA *in situ* hybridization signals (Fig. 5) of the steroidogenic enzymes involved in cortisol biosynthesis were detected in almost all of these adrenocortical nodules, in contrast to the adrenocortical nodules in adrenals with ACTH-dependent bilateral adrenocortical hyperplasia and Cushing's adenoma, which demonstrated marked heterogeneity of expression of steroidogenic enzymes in the lesions [8]. These results indicated that almost all of the cortical cells in the nodules produce cortisol and are associated with an increased production of the enzyme protein, which can also explain the presence of hypercortisolism despite the small sizes of adrenals with PPNAD. The internodular cortex of the adrenals with PPNAD was negative for the enzymes except for sporadic immunoreactivity of 3βHSD, which is also consistent with ACTH-independent hypercortisolism, that is, suppression of the hypothalamo–pituitary–adrenal axis. Immunoreactivity and *in situ* hybridization signals of steroidogenic enzymes are observed in a small cluster of cortical cells with abundant eosinophilic cytoplasms located at the zona reticularis but not in the adjacent non-nodular cortex, which may support an abnormal development of the zona reticularis rather than an exogenous factor in the pathogenesis of the disorders [22].

ACTH-Independent Macronodular Adrenocortical Hyperplasia In addition to PPNAD described in the preceding, an increasing number of cases in which bilateral adrenocortical macronodules are observed in the presence of suppressed serum ACTH levels have been reported recently in the literature [23–27]. These adrenocortical lesions were recently designated AIMAH. AIMAH is clinically characterized by excessive and autonomous cortisol secretion and a markedly enlarged bilateral adrenal gland without ACTH hypersecretion.

Grossly, the adrenal glands are markedly enlarged, ranging from 28 g to 105 g in our series [27] and demonstrated numerous yellow nodules throughout the cortex. Light tan nodules are also observed in some cases. A normal non-nodular adrenal cortex was generally not discernible. The adrenal glands in AIMAH are composed of two different characteristic cell types, clear and compact cells (Fig. 6). Generally, clusters of compact cells are dispersed in the clear cortical cells of the adrenals in AIMAH. Immunoreactivity to $P450_{scc}$, $P450_{c21}$, and $P450_{c11}$ was observed in both clear and compact cortical cells, with compact cells, displaying more intense staining as reported in Cushing's adenoma and ACTH-dependent bilateral adrenocortical hyperplasia [7]. However, immunoreactivity and mRNA hybridiza-

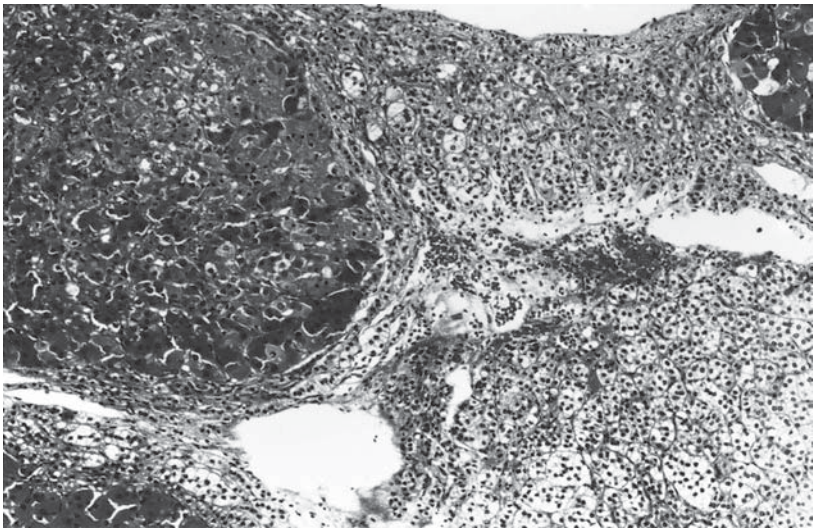

Figure 4 Light microscopic features of primary pigmented nodular adrenocortical disease (PPNAD). Nodules that are composed of relatively large cortical cells with abundant eosinophilic cytoplasm and adjacent atrophic cortex are observed in this field.

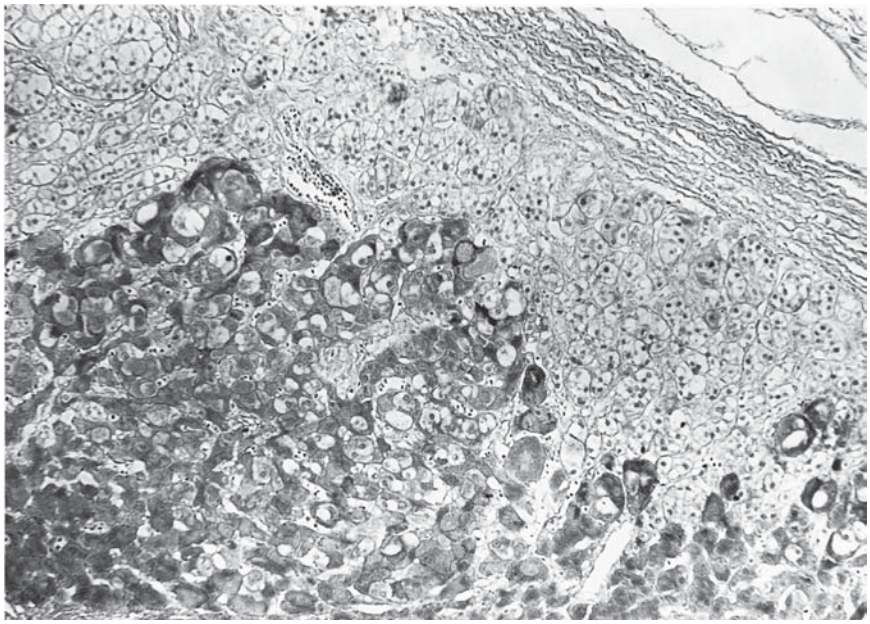

Figure 5 Immunohistochemistry of C17 in the adrenal of PPNAD. C17 or 17α-hydroxylase immunoreactivity was predominantly detected in the nodules composed of cortical cells with abundant eosinophilic cytoplasm.

tion signals of $P450_{c17}$ were observed predominantly in small compact cells, while those of 3βHSD occurred exclusively in clear cortical cells [7,25] (Fig. 7). This differential expression of 3βHSD and $P450_{c17}$ in clear and compact cortical cells has been observed only in AIMAH among the adrenocortical disorders associated with Cushing's syndrome. Further investigations are required to obtain the correlation of this differential expression of 3βHSD and $P450_{c17}$ to cortisol production in adrenals with AIMAH, but distribution of the enzymes is considered to represent ineffective corticosteroidogenesis; that is, progesterone produced as a result of 3β-hydroxysteroid dehydrogenation in the large clear cortical cells may not be effectively converted to cortical cells because $P450_{c17}$ is present only in compact cortical cells. It is considered that this ineffective corticosteroidogenesis may contribute to the relatively low production of cortisol per tissue in AIMAH [8,27].

As described above differential diagnosis between non-neoplastic and neoplastic ACTH-independent hypercortisolism may not be difficult, if surgical pathologists are familiar with specific histological features of non-neoplastic lesions above.

ADRENOCORTICAL INCIDENTALOMA (HORMONALLY INACTIVE ADRENOCORTICAL (ADENOMA) Morphologically, these adrenocortical lesions cannot be differentiated from adrenocortical adenoma associated with Cushing's syndrome or primary aldosteronism [28,29]. Immunohistochemical analysis of steroidogenic enzymes in these adrenocortical incidentaloma demonstrated immunoreactivity of all the enzymes involved in corticosteroidogenesis in tumor cells [28,29], except

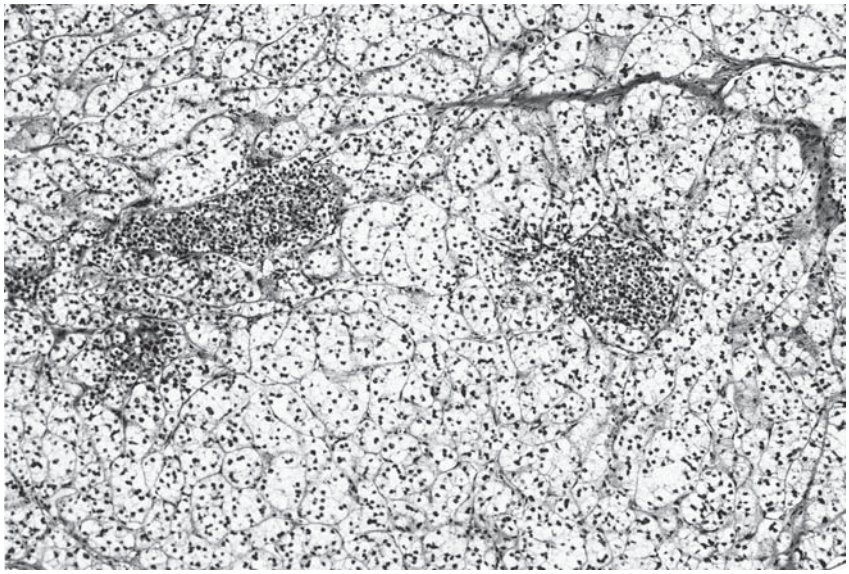

Figure 6 Light microscopic features of ACTH-independent macronodular adrenocortical hyperplasia (AIMAH). Clusters of small cortical cells with scant cytoplasm and relatively large cortical cells with abundant clear cytoplasm were observed in this field.

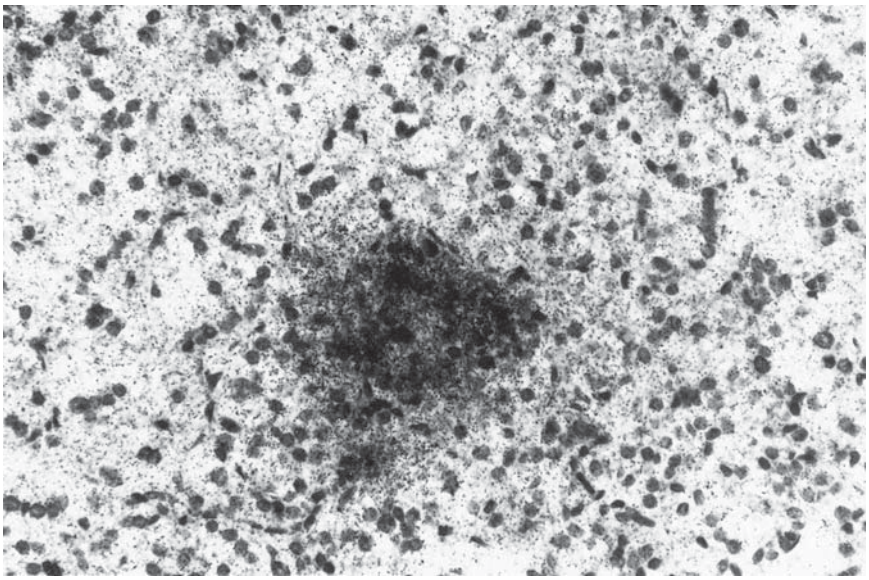

Figure 7 Double immunohistochemistry and mRNA *in situ* hybridization of C17 in the adrenal of AIMAH. Both immuroreactivity and mRNA hybridization signals were predominantly detected in the compact cells.

for adrenocortical oncocytoma [30]. Adrenocortical oncocytoma is composed of compact cells with abundant lipid-sparse eosinophilic cytoplasms (Fig. 8). Electron microscopic examination revealed abundant mitochondria with the occasional presence of intramitochondrial crystals [30]. These findings indicate that a great majority of incidentally detected adrenocortical lesions can synthesize cortisol, but in amounts insufficient to cause hypercortisolism [28]. The only morphological differences between hormonally active and inactive adrenocortical incidentalomas may be the presence or absence and the degree of cortical atrophy of attached non-neoplastic adrenal glands [28,29]. In addition, the degree of cortical atrophy is to some extent correlated with the degree of suppression of serum cortisol by the dexamethasome suppression test and that of diminished expression of steroidogenic enzymes, especially DHEA-ST in the attached non-neoplastic adrenal.

Osella et al. reported a correlation between low plasma levels of DHEA-A and increased cortisol nonsuppressibility by dexamethasone treatment [31]. Therefore serum DHEA-S levels are postulated to reflect the status of the hypothalamo–pituitary–adrenal axis [31]. A single determination of serum DHEA-S levels has been proposed as an easy and useful method for the screening of subtle hypercortisolism in patients with adrenocortical incidentaloma [31]. However, it is very important to recognize that normal serum levels of DHEA-S decrease with age [32,33] and are therefore low in elderly subjects. Therefore,

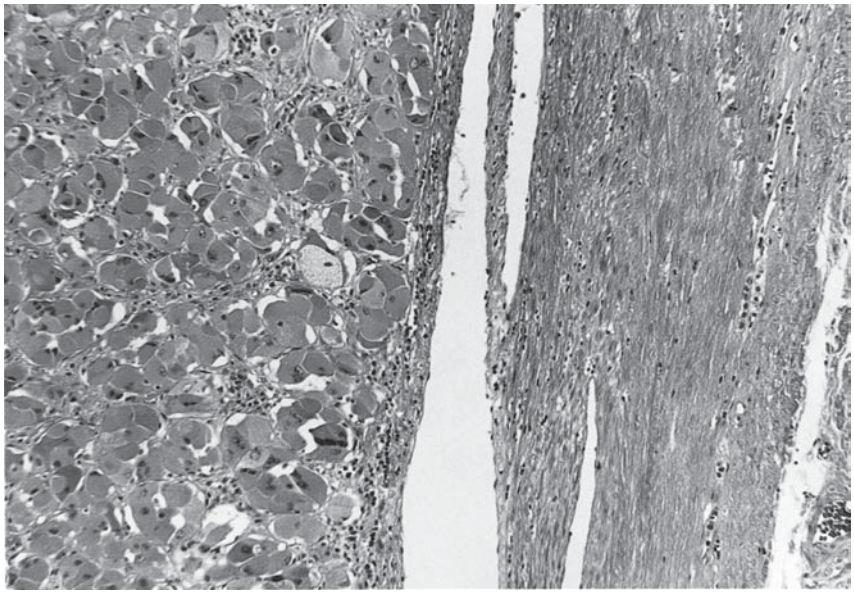

Figure 8 Light microscopic features of adrenocortical oncocytoma. Relatively large tumor cells with abundant eosinophilic cytoplasm were detected in this field. Compressed non-neoplastic adrenal glands were detected inside of the capsule.

serum DHEA-S levels should be carefully evaluated in elderly subjects with adrenocortical incidentalomas. DHEA-ST catalyzes the 3'-phosphoadenosine 5'-phosphosulfate-dependent sulfation of a wide variety of steroids including DHEA. Sulfation of DHEA by DHEA-ST results in the production of DHEA-S. DHEA-ST expression in the human adrenal is also regulated by ACTH [34]. DHEA-ST immunoreactivity was present in almost all the zona reticularis cells and some cortical cells, demonstrating lipid depletion in the zona fasciculata but not in the zona glomerulosa of the normal adrenal. In attached adrenals in cases of adrenocortical neoplasms, especially adrenocortical incidentalomas, the degree of DHEA-ST expression in the zona reticularis of the attached non-neoplastic glands correlated well with that of dexamethasone suppressibility and serum DHEA-S levels. Therefore, it is very important to study not only the presence or absence and/or the degree of cortical atrophy in the attached non-neoplastic adrenal but also DHEA-ST expression in these adrenals, which can contribute to the more precise evaluation of preoperative status of the hypothalamo–pituitary–adrenal axis of these patients. From a practical standpoint, when confirming that the resected adrenocortical mass is grossly considered of adrenocortical origin based on the color of the lesion at the cut surface and others and its attached adrenal shows macroscopic adrenocortical atrophy, prophylactic postoperative glucocorticoid replacement therapy is advised to avoid postoperative adrenocortical insufficiency.

HYPERALDOSTERONISM

Hyperaldosteronism can also be subclassified as primary or secondary based on its etiology. Pathologists rarely receive specimens of secondary hyperaldosteronism caused by an elevated renin–angiotensin system including renovascular hypertension. Primary aldosteronism is also designated as hyperaldosteronism with low plasma renin. Primary aldosteronism is clinically characterized by hypokalemic alkalosis, hypertension, and muscle weakness, which are all caused by elevated plasma aldosterone concentration. In patients with primary aldosteronism, the level of plasma aldosterone concentration is increased and plasma renin concentration is in principle suppressed. Primary aldosteronism can also be subclassified further into neoplastic and non-neoplastic primary aldosteronism, as in ACTH-independent hypercortisolism. The clinical management of patients, including the requirement for adrenalectomy, is different depending on whether the hyperaldosteronism is neoplastic or preneoplastic, as for patients with hypercortisolism. Therefore, numerous clinical studies have been devoted to hormonal differentiation between neoplastic and non-neoplastic hyperaldosteronism, but there have been no single established steroid markers that can reliably differentiate between these two lesions. In principle, neoplastic primary aldosteronism is a unilateral lesion, and non-neoplastic primary aldosteronism is a bilateral adrenocortical lesion, although we experienced five cases of bilateral aldosteronoma in addition to several case report studies [35,36]. In addition, we recently experienced one case of a unilateral adrenocortical nodule producing excessive aldosterone in our consultation series. However, practically, in patients with primary aldosteronism, it is very important to determine the laterity of the lesions through selective venous sampling and radiological examination including CT and magnetic resonance imaging (MRI) scan.

NEOPLASTIC PRIMARY ALDOSTERONISM (ALDOSTERONOMA) The great majority of cases of neoplastic primary aldosteronism are adenomas and carcinomas are very rare. Grossly, aldosteronoma is in general a single unilateral well-circumscribed adrenocortical lesion that may or may not be encapsulated. The size varied from 0.5 cm to 6 cm in our experience. The great majority of aldosteronoma demonstrated a golden-yellow color on the cut surface (Fig. 9), but some cases showed a light to dark tan color on the cut surface. Black adenomas associated with primary aldosteronism are very rare. Foci of necrosis

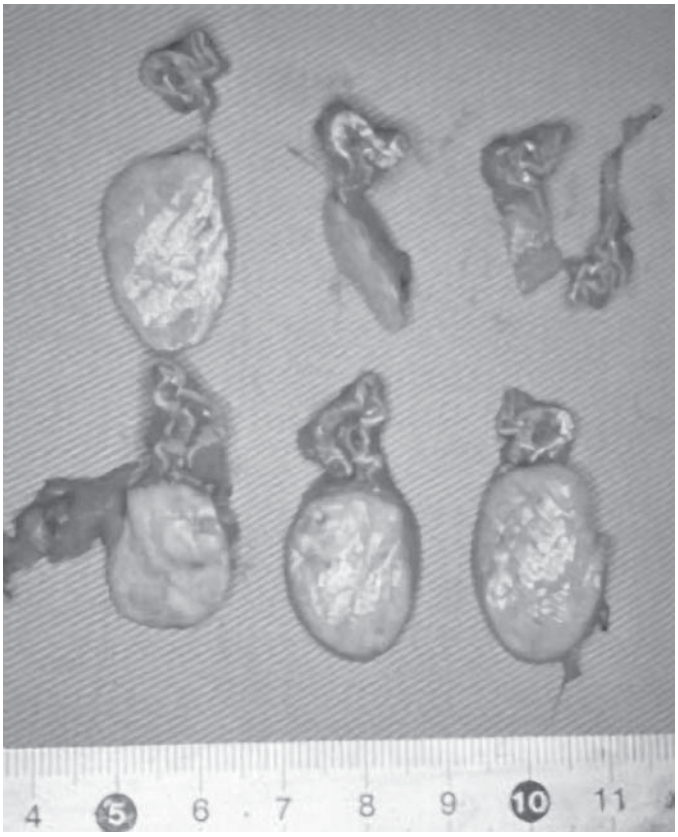

Figure 9 Macroscopic features of aldosteronoma. The cut surface revealed a golden yellow appearance. (Color illustration appears in insert following p. 148.)

or hemorrhage are in principle not detected. Attached non-neoplastic adrenals may exhibit cortical atrophy, as described below. Microscopically, the tumor cells are composed of clear and compact cells. Clear cells can be either large or small. Expression of steroidogenic enzymes is observed predominantly in the compact tumor cells [12]. Aldosteronoma has been considered to be exclusively involved in aldosterone production. However, dexamethasone suppression tests revealed unsuppressibility of plasma cortisol levels [37]. In addition, $P450_{c17}$, which is involved in glucocorticoid and/or androgen synthesis but not in aldosterone biosynthesis, is also expressed in compact cells of some tumors [10,37]. This expression of $P450_{c17}$ demonstrated autonomous neoplastic production of cortisol, as shown by results of a dexamethasone suppression test [37] and occasional cortical atrophy of zonae fasciculata and reticularis of non-neoplastic adjacent adrenal [12,37], due to suppression of pituitary ACTH secretion by excessive autonomous neoplastic cortisol secretion. In the old literature, tumor cells of aldosteronoma were histologically classified as zona glomerulosa, zona fasciculate, and zona reticularis types but this classification does not have any biological significance at this juncture, as the study of $P450_{c17}$ expression demonstrated [12,37]. In addition, the ratio of compact tumor cells was by no means correlated with the patterns or amounts of neoplastic aldosterone secretion or metabolism in our experience.

It is well known that the adrenal cortex adjacent to an aldosteronoma and the contralateral adrenal frequently demonstrated hyperplasia of the zona glomerulosa. In patients with aldosteronoma, the renin–angiotensin system is suppressed and the zona glomerulosa of the attached non-neoplastic adrenals is expected to demonstrate atrophy, as in the zonae fasciculata–reticularis of the attached non-neoplastic adrenal of Cushing's adenoma. Therefore, this hyperplasia of the zona glomerulosa has been termed paradoxical hyperplasia [11–13]. However, the zona glomerulosa cells in these adrenals did not demonstrate the elevated expression of steroidogenic enzymes except for $P450_{c21}$ [4,8]. Therefore, these cells are considered not to be involved in the overproduction of aldosterone. As described below, the absence of overexpression of steroidogenic enzymes in the zona glomerulosa cells of the attached adrenal is very important in the differentiation between idiopathic hyperaldosteronism or non-neoplastic hyperaldosteronism and aldosteronoma [8].

The molecular or genetic basis of aldosteronoma has not been well characterized. We detected overexpression of $P450_{aldo}$ or aldosterone synthetase in aldosteronoma tissues, but this is a quite expected finding considering the pathophysiology of the disorder. Various genetic analyses have so far failed to identify the characteristic genetic changes associated with aldosteronoma. The great majority of aldosteronomas are by no means a genetic disorder, but it is also true that primary aldosteronism due to adrenocortical adenoma or carcinoma can also occur as part of the multiple endocrine neoplasia syndrome, in which loss of heterozygosity has been described on chromosome 11q13, although abnormalities of chromosome 11q13 have not been detected in sporadic or nonfamilial cases of aldosteronoma, which comprise almost all cases of aldosteronoma [38]. Abnormalities of this locus, as well as renin gene restriction fragment length polymorphism have been proposed as one of the possible bases of genetic abnormalities of aldosteronoma by some investigators [38]. However, this, of course, needs to be verified by large-scale analysis. At this juncture, an abnormality of genomic DNA or the presence of specific gene(s) causing sporadic or nonfamilial cases of aldosteronoma is considered a remote possibility. The majority of genetic abnormalities are considered to reflect the results of neoplastic transformation.

NON-NEOPLASTIC PRIMARY ALDOSTERONISM Non-neoplastic primary aldosteronism is subclassified into idiopathic hyperaldosteronism (IHA) and dexamethasone suppressible hyper-aldosteronism (DSH) or glucocorticoid suppressible hyperaldosteronism (GSH). Both IHA and DSH are in principle bilateral adrenocortical lesions. Clinical and hormonal features of IHA are essentially the same as those of aldosteronoma. Less severe hypokalemia, resistance to spironolactone treatment, enhanced aldosterone secretion in response to angiotensin, and others have been reported as characteristics rather of patients with IHA [13,39–41]. However, as described previously, these hormonal features of the patients are helpful but not diagnostic for IHA. In addition to clinical and hormonal features of primary aldosteronism, DSH is characterized by remediability by long-term administration of glucocorticoids [42–44]. DSH is known as an autosomal dominant disease [45] and molecular mechanisms of this adrenocortical disease has been elegantly clarified by recent investigations. The underlying genetic defect

of this disease is the presence of a hybrid gene in which 11β-hydroxylase gene regulatory elements are fused to the coding region of the aldosterone synthetase gene through unequal meiotic cross over [46–49]. Therefore, in patients with DSH, this chimeric or hybrid gene encodes a fused P-450 protein consisting of the N-terminal side of the 11β-hydroxylase gene and the C-terminal side of the aldosterone synthetase gene [47]. The expression of this gene is regulated like 11β-hydroxylase but can also result in the synthesis of aldosterone [46–49]. Therefore, in patients with DSH, aldosterone biosynthesis is under the control of ACTH and remediable by dexamethasone treatment, which can suppress pituitary ACTH secretion and subsequently aldosterone secretion from the adrenal. In contrast to DSH or GSH, the molecular and cellular etiologic bases of IHA have not been established.

Bilateral or unilateral adrenalectomy used to be performed in patients with IHA to alleviate the symptoms of hyperaldosteronism. However, recently, adrenalectomy has rarely been performed on patients clinically diagnosed with IHA or DSH, and these patients are treated medically. Patients with DSH should be treated with glucocorticoid administration. Therefore, it is unlikely for surgical pathologists to examine the adrenal glands of patients who are clinically diagnosed with IHA or DSH. However, it is still very important to differentiate IHA, especially that associated with adrenocortical nodular hyperplasia, from aldosteronoma, particularly bilateral aldosteronoma. In addition, the number of very small (0.2–0.5 cm) aldosteronoma submitted to our consultation files increased recently because of the improved resolution of the CT or MRI scan and increased frequency of applying these radiological diagnostic procedures to patients with hypertension with mildly elevated plasma aldosterone concentration in some institutions. Selective venous sampling of plasma aldosterone concentration of adrenal veins is effective in determining the laterality in most cases.

Differentiation Between IHA and Aldosteronoma As previously described, it is sometimes difficult to differentiate clinically between IHA and aldosteronoma, for the following reasons: (1) aldosteronoma can be bilateral; (2) IHA patients may manifest a unilateral macronodule that can be detected by CT or MRI scan; and (3) the contralateral adrenal of aldosteronoma frequently demonstrated adrenocortical nodule(s). In our experience, the third one is the most frequently encountered problem. As described above, it is true that the selective adrenal venous sampling of aldosterone is usually effective in the clinical diagnosis. Adrenocortical nodules in patients with aldosteronoma are more frequently detected in patients with a history of longstanding hypertension, which caused nodule formation in the non-neoplastic adrenal in our experience. Histological features of macronodules in adrenals of patients with IHA can be similar to those of aldosteronoma, that is, a combination of clear and compact cortical cells. The zona glomerulosa can be morphologically hyperplastic in the adrenals of both IHA and aldosteronoma patients. Therefore, it is not necessarily easy to differentiate IHA from aldosteronoma even by histological examination in some cases of primary aldosteronism. However, histological differentiation between IHA and aldosteronoma in the resected adrenal is very important because unilateral adrenalectomy generally relieves hypertension in patients with aldosteronoma but not necessarily in those with IHA. The etiology of paradoxical hyperplasia is unknown but the morphologically hyperplastic zona glomerulosa is not considered to be involved in complete aldosterone biosynthesis as was described above. Immunolocalization of steroidogenic enzymes demonstrated that the zona glomerulosa cells in the adrenals with IHA exhibited marked immunoreactivity of all the enzymes except for $P450_{c17}$ (see Fig. 18A), while those in the non-neoplastic adrenals with aldosteronoma did not have increased expression of the enzymes except for $P450_{c21}$. It is therefore concluded that immunolocalization of steroidogenic enzymes, especially that of 3βHSD is considered as the only reliable diagnostic method of differentiating IHA from aldosteronoma in the resected adrenals of some patients with primary aldosteronism.

DISCERNING MALIGNANCY IN RESECTED ADRENOCORTICAL LESIONS

When patients with an adrenocortical mass or with adrenocortical dysfunctions are clinically detected, the most important clinical aspects in their management is whether that adrenal mass represents malignant neoplasm or whether or not adrenocortical dysfunction is caused by adrenocortical carcinoma. It is also true that the most important and cardinal point in adrenocortical pathology is the differential diagnosis between adrenocortical adenoma and carcinoma. In this section, gross and histopathological findings of adrenocortical carcinoma pertinent to differential diagnosis as well as cellular and molecular findings that may contribute to differential diagnosis of adrenocortical malignancy are summarized.

MACROSCOPIC When evaluating malignancy of resected adrenocortical neoplasms, macroscopic observation of the specimen submitted to diagnostic pathology laboratories is very important. The first important factor is the weight of the tumor. Therefore, the weight of the neoplasm should be determined as carefully as possible when evaluating an adrenocortical neoplasm. In our experience of 66 cases, tumors weighing 100 g comprised 93% of carcinoma but only 6% of the adenoma. Tang and Gray reported that all cortical tumors weighing >95 g were malignant, whereas tumors <50 g in weight were benign (the average weight of the tumor is 705 g ranging from 96 to 2460 g) [50]. Van Slooten et al. reported that in their series only tumors weighing >150 g metastasized [51]. However, it is also important to note that small adrenocortical tumors can metastasize and some large tumors do not. The tumor reported by Gandour and Grizzle weighed only 40 g and measured only 4 cm in greatest dimension but metastasized 3 yr following bilateral adrenalectomy [52]. On the other hand, Hough et al. reported that the tumor weighing 1800 g did not metastasize [53]. Therefore, the weight of the tumor is important in evaluating malignancy of adrenocortical neoplasms, but the weight itself is not a reliable prognostic indicator of the resected adrenocortical tumor.

The next important factor is the macroscopic features of the cut surface of the tumor. Hemorrhage and necrosis are rarely observed in adrenocortical adenoma. Necrosis is sometimes associated with cystic degeneration. The presence of necrosis

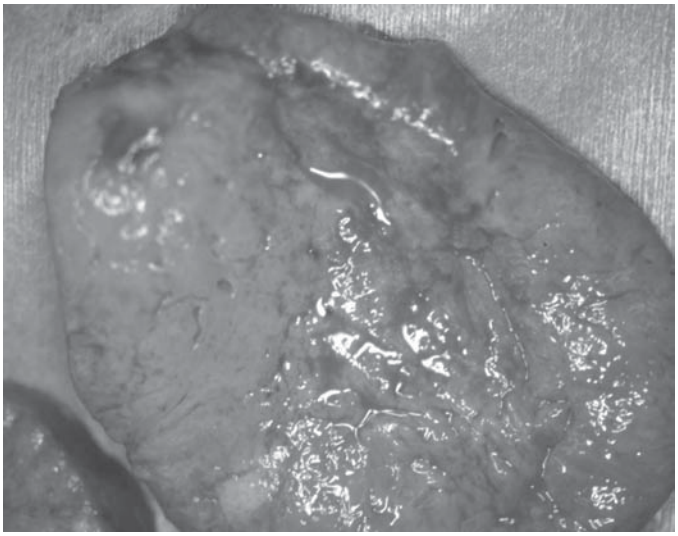

Figure 10 Macroscopic features of adrenocortical carcinoma. Only the specimens sampled from the area adjacent to necrosis and hemorrhage were diagnostic as adrenocortical carcinoma. (Color illustration appears in insert following p. 148.)

and hemorrhage therefore strongly indicates the diagnosis of adrenocortical carcinoma. However, it is also true that many adrenocortical carcinoma were not associated with foci of necrosis and hemorrhage. In addition, it is also important to sample the specimens from the areas adjacent to the foci of necrosis and hemorrhage when preparing gross specimens (Fig. 10). It is important to note that foci of intratumoral fibrosis and myxamatous degeneration [54] can be seen in both adenoma and carcinoma and adrenocortical carcinoma may be well circumscribed and encapsulated. The color of the cut surface of viable parts of adrenocortical neoplasms is not a reliable indicator of adrenocortical carcinoma. Carcinoma may be tan, yellow, or yellow orange but a homogeneous black cut surface, as observed in black pigmented adenoma, is rarely observed in adrenocortical carcinoma.

HISTOLOGICAL DIFFERENTIATION BETWEEN ADRENOCORTICAL ADENOMA AND CARCINOMA It is true that a large number adrenocortical carcinomas are associated with characteristic gross features described above, including large size, necrosis, and hemorrhage, and do not usually pose diagnostic problems. However, an increasing number of small adrenocortical neoplasms has been discovered with the development of CT and MRI scans. Therefore, adrenocortical carcinomas not associated with these ominous macroscopic features have recently increased in number. The distinction of these "well-differentiated" adrenocortical carcinomas from adenoma may be one of the greatest diagnostic difficulties in surgical pathology practice. There is no single histological criterion that can reliably differentiate adrenocortical carcinoma from adenoma-like capsular or vascular invasion of thyroid follicular carcinoma. Only the systems that evaluated multiple histological and/or nonhistological criteria of the resected cases can provide reliable histological diagnosis. Three different histological scoring systems have been proposed by various investigators [51, 53,55,56] and all systems are equally useful for predicting clinical outcome of patients with resected adrenocortical neoplasms.

Table 1
Weiss System

1. High nuclear grade
2. More than five mitotic figures per 50 high-power fields
3. Atypical mitotic figures
4. Eosinophilic or compact tumor cell cytoplasm (>75% of tumor cells)
5. Diffuse architecture (>33% of tumor)
6. Necrosis (confluent necrosis)
7. Venous invasion (smooth muscle in wall)
8. Sinusoidal invasion (no smooth muscle in wall)
9. Capsular invasion

Table 2
Van Slooten System

Histologic criteria	*Numerical value*
1. Extensive regressive changes	5.7
2. Loss of normal structure	1.6
3. Nuclear atypia	2.1
4. Nuclear hyperchromasia	2.6
5. Abnormal nucleoli	4.1
6. More than two mitotic figures per 10 high-power fields)	9.0
7. Vascular or capsular invasion	3.3

Table 3
Hough System

Histologic criteria	*Numeric value*
1. Diffuse growth pattern	0.92
2. Vascular invasion	0.92
3. Tumor cell necrosis	0.69
4. Broad fibrous bands	1.00
5. Capsular invasion	0.37
6. Mitotic index (more than 1 per 10 high-power fields)	0.60
7. Pleomorphism	0.39
Nonhistologic criteria	
1. Tumor mass (>100 g)	0.60
2. Urinary 17-ketosteroids (10 mg/g creatinine/24 h)	0.50
3. Response to ACTH (17-hydroxysteroids increased two times after 50 μg of ACTH IV)	0.42
4. Cushing's syndrome with virilism, virilism alone, or no clinical manifestations	0.42
5. Weight loss (more than 10 pounds/3 mo)	2.00

They are summarized in Tables 1–3. Hough and his colleagues proposed 12 criteria, 7 histologic and 5 nonhistologic, of predicting clinical outcome of the patients by studying 41 cases of adrenocortical tumors [53]. A numerical value for these12 criteria was determined by employing a modified Bayes theorem for predicting the possibility of metastasis. When assessing an individual case, these numerical values are combined and the histologic and nonhistologic indices were subsequently determined. The combination of histologic and nonhistologic indices was reported to effectively differentiate adrenocortical carcinoma from adenoma [53]. The criteria were very useful in

predicting subsequent biological behavior of resected adrenocortical tumor once familiarity with the system is gained. In addition, the inclusion of clinical findings is considered to enhance the reliability of the diagnosis. Nevertheless, this system may be limited by the requirement of clinical findings, although results of these findings must be available for pathologists, and possibly the relatively complicated numerical processes.

Van Slooten et al. developed a similar scoring system using seven histologic criteria of the resected neoplasms (Table 2) with assigned numerical values for differentiating adrenocortical carcinoma from adenoma [51]. The numerical values for these severe histologic parameters described in Table 2 were subsequently combined and a histologic index for that case was obtained. A histologic index >8 is considered to correlate with aggressive biological behaviors. When we retrospectively applied these van Slooten criteria to resected adrenocortical tumors, some adrenocortical adenomas were erroneously diagnosed as carcinoma. However, the evaluation of these histological features is straightforward, and it is relatively easy to apply this system in histological diagnosis.

In 1984, Weiss proposed nine histological criteria (Table 3) important in evaluating adrenocortical malignancy [55]. Weiss subsequently lowered the threshold for adrenocortical malignancy from four to three histologic criteria because 20 of 23 cases that fulfilled three histologic criteria died of disease [56]. The system is straightforward and relatively easy to use, and a good correlation can be detected between results and clinical outcome of the patients. However, it is also true that the tumors that behaved in a non-malignant fashion in their postoperative course, including cases of adrenocortical oncocytoma [57], were considered as adrenocortical carcinoma, although these adrenocortical oncocytomas may recur or metastasize over a long period of time. In addition, among these nine criteria, we experienced that nuclear grade, architecture, and cytoplasm were likely to be subjective, that is, the interobserver differences were relatively marked unless observers were well informed prior to histological examination of adrenocortical tumor.

In general, it is important to combine gross features including those described previously and the aforementioned histological scoring systems above to reach the diagnosis of adrenocortical carcinoma. The value of histological criteria and gross features of the resected specimens for differentiating adrenocortical carcinoma from adenoma in pediatric cases is more complicated than for adult cases. In our experience, adrenocortical tumors histologically diagnosed as carcinoma based on the criteria described above turned out to behave less aggressively compared to adult cases. This is possibly because the tumor is more likely completely excised or the intrinsic biological behavior of the tumor itself is less aggressive in children. However, the combination of gross features and histological criteria described above is still considered to be reasonably effective in making the diagnosis of malignancy in pediatric adrenocortical neoplasms.

MOLECULAR AND CELLULAR FEATURES OF ADRENOCORTICAL CARCINOMA Recently, the application of molecular and cellular biology and molecular tools in cancer research yielded a new dimension in our understanding of human cancer. However, they are not necessarily well studied compared to other human malignancies. Relatively rare frequency of adrenocortical carcinoma prevents investigators from drawing definitive conclusions about biological significance of the results obtained from molecular and cellular studies. In addition, there are no established premalignant conditions in the human adrenal cortex and the transition from adrenocortical adenoma to carcinoma has not been well documented. Therefore, the possible significance or roles of the molecular and cellular abnormalities detected in carcinoma patients in tumorigenesis or carcinoma development can be very difficult to evaluate. In addition, human adrenocortical carcinomas are markedly heterogeneous in morphology and biological function even within the same tumor. Thus, it is very important to note that molecular and cellular features of human adrenocortical carcinoma are clinically of no value and/or significance even if meticulously and elegantly performed unless the findings are correlated with morphological features. Therefore, in this section, we will summarize recent developments in molecular and cellular features of adrenocortical carcinoma with emphasis on the possibility of applying those to the evaluation of the differences between adrenocortical adenoma and carcinoma and/or of the biological behavior of the resected neoplasms as auxiliary diagnostic means.

DNA Content Adrenocortical neoplasms that recurred or metastasized were reported to be more likely to demonstrate DNA aneuploidy than those showing no evidence of further disease during the postoperative follow-up period [58]. We also reported that seven of eight adrenocortical carcinomas demonstrated DNA aneuploidy while all adenomas were diploid by flow cytometry [59]. However, a number of studies also reported that 20–40% of adrenocortical adenomas have DNA aneuploidy and a small subset of carcinomas were diploid [60–62]. In addition, Camuto et al. demonstrated that there was no correlation among ploidy status and survival, response to therapy, or steroid hormone production in adrenocortical neoplasms [63]. Therefore, the value of DNA ploidy in determining biological behavior of resected adrenocortical neoplasms is still in dispute and further studies are required to establish it as a possible auxiliary mean of evaluating adrenocortical neoplasms.

Cell Proliferation Cell kinetic information is becoming a valuable adjunct to histopathologically based tumor classification [64]. Among the various methods used to assess cell proliferation or cell kinetics in surgical pathology specimens submitted to diagnostic pathology laboratories, immunohistochemical analysis of cell-cycle-related antigens has advantages over other conventional methods.

The monoclonal antibody Ki-67 is considered to recognize a nuclear antigen present in all phases of the cell cycle while the proliferating cell nuclear antigen (PCNA) is an auxiliary protein of DNA polymerase δ and is associated with the late G_1 and S phases of the cell cycle. The availability of MIB-1 in combination with antigen retrieval made it possible to perform Ki-67 immunostaining in 10% formalin-fixed and paraffin-embedded materials (Fig. 11). The Ki-67 labeling index of adrenocortical carcinoma was reported to be significantly higher than for adrenocortical adenoma by our groups [61] and the group of Lloyd [65]. On the other hand, PCNA labeling index was not significantly different between adrenocortical adenoma and carcinoma

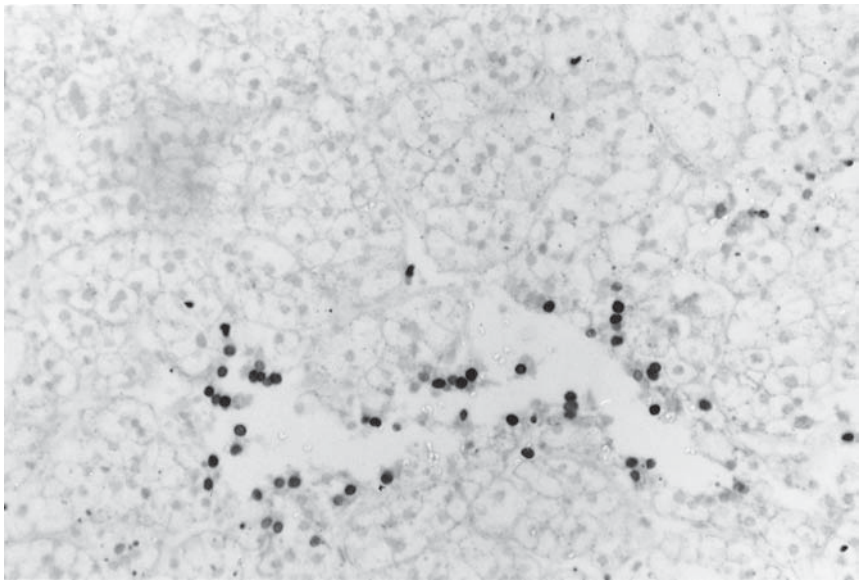

Figure 11 Immunohistochemistry of Ki-67 in adrenocortical carcinoma.

[59,65], as was reported in other human malignancies. In our study of immunohistochemical evaluation of Ki-67 in human adrenocortical neoplasms, 11 of 17 carcinomas demonstrated a labeling index of more than 2.5, whereas none of the adenomas did [66]. Therefore, resected adrenocortical neoplasms with a labeling index >2.5 may represent adrenocortical carcinoma. Therefore, Ki-67 immunostaining is of value in differentiation between adrenocortical adenoma and carcinoma and may be incorporated into the histological evaluation of adrenocortical neoplasms, especially histologically intermediate cases. However, as is well known, it is also important to note that inter- or intraobserver differences can become problems when applying the Ki-67 labeling index to surgical pathology differential diagnosis between adrenocortical adenoma and carcinoma. These differences can frequently be experienced through evaluation of Ki-67 immunostain of resected neoplasms in various laboratories. This is due to (1) uneven distribution of Ki-67 immunoreactivity, that is, how many fields it is necessary to count and (2) interpretation of weak nuclear immunoreactivity, that is, the threshhold of positivity when we apply the Ki-67 labeling index to resected adrenocortical carcinoma. In our laboratory, we select at least 10 fields and count at least 500, preferably 1000 tumor cells. Even when we use a computer image analyzer for evaluation of Ki-67 immunohistochemistry, the selection of the fields for counting and the threshhold of nuclear positivity can still be a problem.

Growth Factors Overexpression and/or other abnormalities of various growth factors have been demonstrated to be associated with aggressive biological behavior in many human malignancies. In human adrenal and adrenocortical disorders, growth factors have been examined for their possible roles in modifying corticosteroid production and/or secretion through evaluation of adrenocortical free cell preparation. However, abnormalities of growth factors have not necessarily been well studied in human adrenocortical carcinoma, compared to other malignancies. Recently, overexpression of transforming growth factor-α and epidermal growth factor receptor was demonstrated in adrenocortical carcinoma cases [67]. Elevated expression of insulin-like growth factor (IGF) II was reported in functioning adrenocortical carcinoma [68]. Very recently, Wilkin et al. demonstrated that induction of the phenotype of the fetal adrenal cortex by IGF-2 overexpression and steroidogenesis as well as defective apoptosis may be the cause of pediatric adrenocortical tumors [69]. IGF-I overexpression was also reported in human adrenocortical carcinoma [70]. Inhibins and activins are dimeric proteins of the transforming growth factor-β superfamily. They were demonstrated to be present in human adrenal cortex and its disorders [71–73]. Munro et al. recently reported that loss of the inhibin α-subunit may be involved in the progression of adrenocortical carcinoma [71]. Fetsch et al. reported that immunocytochemistry of anti-α-inhibin can reliably differentiate between adrenocortical and renal cell carcinoma in fine needle aspiration specimens [74]. However, Arola et al. very recently reported no significant differences of inhibin α expression between benign and malignant adrenocortical tumors [73] and further investigations are required for clarification of possible roles of inhibins/activins in the development and progression of adrenocortical neoplasms. Recently, Martinerie et al. reported that NOVH, which belongs to the CCNCCTGF/CYR61/NOV family of proteins, some of which have chemotactic, mitogenic, adhesive, and angiogenic properties, could be involved in human adrenocortical tumor development [75]. Boccuzzi et al. also reported that the association between TGF-β_1 expression and active steroid secretion is lost in adrenocortical carcinoma [76]. Murray et al. very recently demonstrated the decrement of α_1-connexin 43 gap junctions, which suggests that an analysis of gap junction protein may be of use in the differential diagnosis between adrenocortical adenomas and carcinomas [77].

Cytogenetics Etiology or the mechanism of tumorigenesis of human adrenocortical carcinoma is unknown but it appears that susceptibility to adrenocortical carcinoma seems to be inher-

ited in some individuals or families. Children with Beckwith–Wiedemann syndrome, a very rare growth disorder characterized by macroglossia, gigantism, and omphalocele [78,79], have an increased incidence of tumors, including adrenal adenomas and adrenocortical carcinomas [80,81]. Genetic abnormalities affect the chromosome region (11p15) in these patients [82]. Abnormalities of the *TP53* gene have been reported in these patients. Adrenocortical carcinoma is part of a constellation of tumors inherited in the sarcoma, breast, lung, and adrenocortical carcinoma syndrome described by Li and Fraumeni [83] and Lynch et al. [84], called Li–Fraumeni syndrome, which is also very rare. These observations suggest that some carcinoma, although small in number, are considered to occur as a result of spontaneous transformation of adrenocortical cells by spontaneous mutations of genomic DNA. Various studies have suggested that loss of heterozygosity at loci on the short arm of chromosome 11 (11p) may be important in the pathogenesis of both benign and malignant adrenocortical neoplasms [85]. Yano et al. demonstrated that loss of alleles on chromosome 11p, 13q, and 17p was observed in both primary and metastatic adrenocortical carcinomas but not in adrenocortical adenomas [85]. A breakpoint of 11p13, as well as loss of heterozygosity of alleles on 11p15, has recently been reported in adrenocortical carcinoma cases [86]. Therefore, abnormalities of chromosome 11p are reasonably considered to be involved in tumorigenesis of adrenocortical carcinoma. Dohna et al. recently reported results of comparative genomic hybridization (CGH) analysis in human adrenocortical neoplasms [87]. Adenomas and carcinomas both demonstrated chromosomal imbalances but several chromosomal gains, especially the high-level amplifications, were almost exclusively detected in adrenocortical carcinoma. Zhao also reported that the most frequent DNA copy number changes in adrenocortical carcinomas were losses of 1p21–31, 2q, 3p, 3q, 6q, 9p, and 11q14-qter as well as gains of 17p,17q, and 9q32-qter in their CAH study [88]. These authors postulated that oncogenes determining the early tumorigenesis of adrenocortical tumors may exist on chromosome 17 [88]. Russell et al. reported that changes in chromosomes 3, 9, and X are early events in adrenocortical tumorigenesis, with increasing chromosomal instability with tumor progression [89]. These inconsistent results of CAH analysis reemphasized heterogeneity of human adrenocortical neoplasms.

Recently germline mutations of the *p53* tumor suppressor gene has been implicated in the etiology of this disorder [92]. The germline mutations detected in Li–Fraumeni syndrome appear to be clustered in exon 7 of the *p53* gene and have single-base substitutions resulting in amino acid changes, although a wide range of germline *p53* mutations may be inherited [90]. Subsequent studies revealed that germline *p53* mutations were also found in cancer-prone individuals that were not otherwise indicative of the Li–Fraumeni syndrome [91,92]. Therefore, it is interesting to know whether germline *p53* mutations are present or not in sporadic adrenocortical carcinoma, which comprises the great majority of carcinoma cases. Wagner et al. recently reported that three of six children with adrenocortical carcinoma were found to carry germline *p53* mutations in exons 5, 6, and 7, respectively [93]. Barzon et al. recently reported that mutations in the *TP53* gene are frequent in adrenocortical carcinomas [94]. However, it is also important to note that the patients with adrenocortical carcinoma, except possibly for specific pediatric cases, are by no means prone to the development of other primary malignancies and familial cases of adrenocortical carcinoma are rare. Therefore, the great majority of cases with sporadic adult adrenocortical carcinoma are considered not to harbor germline mutations of *p53* but further investigations are awaited for clarification. It is also important to note that *p53* gene abnormality is one of the most common genetic alterations detected in human malignancies. Therefore, it is important to know whether *p53* abnormalities are detected or not in adrenocortical carcinoma tissues. Reincke et al. reported the relative low prevalence of *p53* abnormalities, that is, 3 out of 11 cases demonstrated *p53* abnormalities, although none of five adrenocortical cases demonstrated *p53* abnormalities [95]. McNicol et al. reported that abnormal *p53* expression did not appear to have any significant prognostic effects in carcinoma [96]. We also could not detect any *p53* abnormalities including overexpression of p53 nuclear protein and *p53* DNA mutations in 10 sporadic adult adrenocortical carcinoma cases. Therefore, in contrast to relatively close association of adrenocortical carcinoma with germline *p53* mutations in some pediatric cases, *p53* abnormalities do not appear to play important roles in the tumorigenesis or development of the majority of adrenocortical carcinoma. Abnormalities of other oncogenes or tumor suppressor genes have not been studied in detail. Suzuki et al. reported altered intracellular localization of a c-*myc* oncogene product in adrenocortical carcinoma [59] but further investigations are needed to clarify the practical importance of the findings. Gortz et al. reported that inactivating mutations of the *MEN1* tumor suppressor gene appear not to play roles in the development of sporadic adrenocortical neoplasms [97]. Heppner et al. also reported that the majority of seven adrenocortical carcinoma cases examined were associated with 11q13 loss of heterogeneity, in which the *MEN1* gene is located, but somatic *MEN1* mutations within the *MEN1* coding region were rare events [98]. Nakazumi et al. reported the possible involvement of decreased expression of *p27*, a cell cycle inhibitor, in the biological behavior of adrenocortical neoplasms [99]. Pilon et al. demonstrated an important role of inactivation of the *p16* tumor suppression gene in the pathogenesis of human adrenocortical tumor [100]. Hirano et al. reported the possible correlation between telomerase activity and biological behavior of adrenocortical neoplasms [101]. Results of these studies employing molecular and cellular biological tools all pointed out the importance of abnormal cell proliferation in the development and progression of adrenocortical carcinoma. However, it is also important to evaluate the properties of invasion and metastasis of adrenocortical neoplasms in assessing biological behavior of resected adrenocortical neoplasms but little has been examined in this field. Further investigations may contribute greatly to our understanding of adrenocortical neoplasms.

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12 Adrenal Medulla and Paraganglia

ANNE MARIE MCNICOL, MD, FRCPATH

INTRODUCTION

Paraganglia are neuroendocrine organs, composed mainly of cells derived from the neural crest that secrete catecholamines or indolamines and peptides. They comprise two groups: those associated with the sympathetic and those with the parasympathetic nervous systems. Sympathetic paraganglia lie in the paraaxial region of the trunk close to the paravertebral and prevertebral ganglia or in the connective tissue adjacent to pelvic organs and include the adrenal medulla. They secrete catecholamines in response to sympathetic neural stimulation. Parasympathetic paraganglia lie in the head and neck and close to vascular structures and branches of the glossopharyngeal and vagus nerves. They include the carotid body. Their function is as chemoreceptors responding to changes in oxygen pressure. Some of these effects occur through loops involving the central nervous system, but the exact mechanisms remain to be elucidated.

The main group of tumors arising from these organs is the paragangliomas, but these have been given a variety of names. Some refer to all sympathetic paragangliomas as pheochromocytomas, subdividing them into intraadrenal or extraadrenal types. Parasympathetic paragangliomas have been defined as chemodectomas, glomus tumors, or nonchromaffin paragangliomas. The current preferred approach is to use the term pheochromocytoma only for intraadrenal tumors and to define the others as paragangliomas, further subdivided on the basis of type (sympathetic or parasympathetic) and site.

In this chapter, general historical aspects, embryology, anatomy, and function of both types are considered together. Tumors are discussed under the separate headings of pheochromocytoma, extraadrenal sympathetic paragangliomas, and parasympathetic paragangliomas. Specific aspects of structure and function of individual paraganglia are included in these sections. Tumors with neuronal differentiation, such as neuroblastoma and ganglioneuroma, are outside the scope of this chapter.

HISTORICAL ASPECTS

Kohn [1] first put forward the concept of a paraganglionic system at the beginning of the last century. He was also the first to use the terms "chromaffin reaction" for the brown color change that occurred in the adrenal medulla on immersion in chromate salts and "chromaffin cells" for cells that underwent this change. He described deposits of chromaffin tissue in extraadrenal retroperitoneal locations. He also confirmed the finding of Stilling [2] that the carotid body contained some chromaffin cells. He therefore proposed that all of these tissues were linked to the sympathetic nervous system and resembled, but were not, ganglia, and should be named paraganglia. The discovery that the innervation of the carotid body was mainly parasympathetic and the demonstration that most of the cells of the carotid body are negative for the chromaffin reaction questioned the concept. This led to a modification in which paraganglia were defined as chromaffin (associated with the sympathetic nervous system), nonchromaffin (associated with the parasympathetic system), or of mixed type [3]. The contrast between the classical endocrine role of the adrenal medulla and the chemoreceptor role of the nonchromaffin paraganglia further challenged a common classification. However, modern methods of investigation have upheld the theory of linkage by demonstrating that the neuroendocrine cells of both types of paraganglia originate from the neural crest and that all can produce catecholamines. The nonchromaffin staining of some cells is related to the production of low levels of catecholamines that cannot be detected by this relatively insensitive technique. It is recommended that the technique should now be recognized as part of the history of paraganglia and should no longer be applied in either diagnostic practice or experimental investigation.

The adrenal glands were first described by the Italian anatomist Bartholomaeus Eustachius in the 16th century [4]. Kölliker [5] described the histology of the glands and put forward the idea that the medulla was related to the nervous system. Addison first identified the essential requirement of adrenocortical function for life [6]. The role of the medulla was first elucidated in 1894 when an adrenal extract was injected into a dog and showed a pressor response [7]. The pressor agent was isolated and characterized and called "epinephrine" [8] or "adrenalin" [9]. Norepinephrine was characterized later [10]. The recognition in the early 1950s that catecholamines were associated with granules in the adrenal medulla [11,12] started the whole scientific investigation of the neurosecretory granule.

The human carotid body was first described in 1743 by Taube and illustrated by Neubauer in 1772. These findings and other

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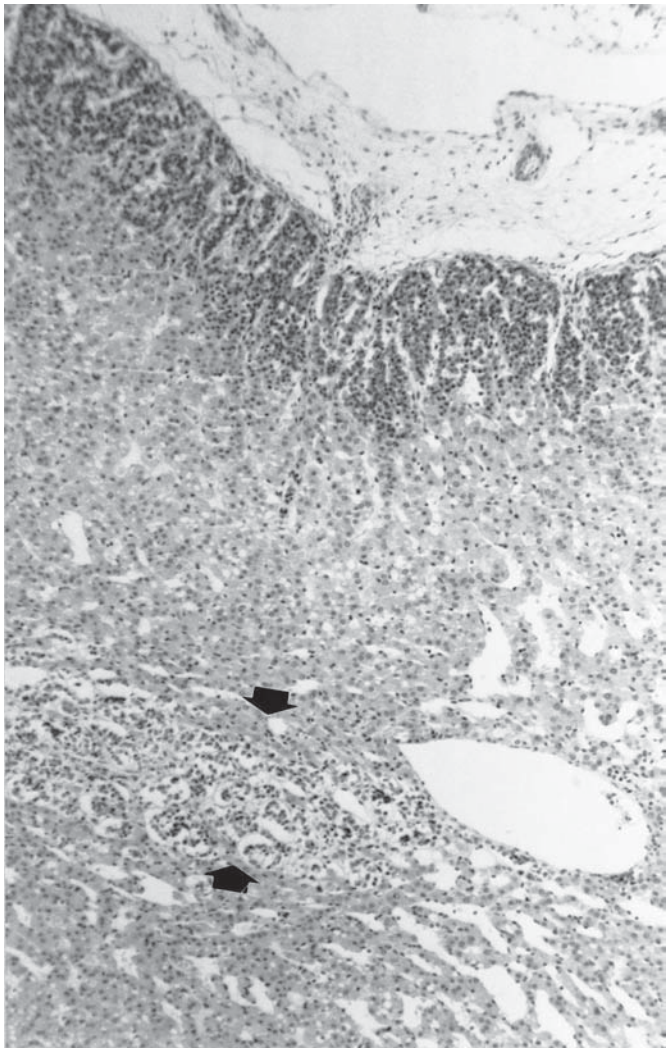

Figure 1 Fetal adrenal gland showing primitive adrenal medulla (*between arrows*) sitting close to the central vein.

historical aspects of paraganglia are extensively reviewed by Lack [13].

NORMAL STRUCTURE AND FUNCTION

EMBRYOLOGY Paraganglia are recognized in the human fetus by 7 wk of gestation, at which time they comprise small primitive cells. These give rise to the neuroendocrine cells, neural cells, and glial (sustentacular) cells. In extraadrenal paraganglia, differentiated cells replace primitive cells by wk 25, but primitive cells persist for longer in the adrenal medulla. In the medulla the primitive cells are originally in aggregates in the cortex, but then migrate to the area around the central vein (Fig. 1).

NORMAL DISTRIBUTION Sympathetic paraganglia are found in close relationship to the peripheral sympathetic nervous system, from the level of the superior cervical ganglion down the sympathetic trunk and into the pelvis. They include the adrenal medullae and the organs of Zuckerkandl around the origin of the inferior mesenteric artery. In the pelvis, they are found in greatest numbers in association with the inferior hypogastric plexi entering the urogenital organs, in the bladder wall, and in the sacral plexus [14]. In the adult, the adrenal medulla is the largest of these. However, in the fetus and up to about 3 yr of age, the organs of Zuckerkandl predominate. These may lie on either side of the aorta or form a fused body lying across it. They later undergo "disintegration," and in the older child and adult, the remnants are usually detected only by microscopy [15]. Sympathetic paraganglia may also be found around the hilum of the kidney and in periadrenal fat, and sometimes in the thoracic region. Neuroendocrine cells may also be found within sympathetic ganglia.

Parasympathetic paraganglia have a more restricted distribution, and are found almost exclusively in association with the thoracic and cranial branches of the glossopharyngeal and vagus nerves. The tympanic paraganglia in the middle ear and the carotid bodies are associated with the glossopharyngeal nerve. The jugular paraganglia of the middle ear, superior and inferior laryngeal paraganglia, subclavian paraganglia, and aorticopulmonary and cardioaortic paraganglia at the base of the heart are innervated by the vagus. Paraganglia of this group may be found within the interatrial septum [16]. The carotid body consistently lies above the carotid bifurcation, but other parasympathetic paraganglia are variable in specific localizations within an anatomical area and in number. Intravagal paraganglia may also be found within or close to the nerve trunk in relation to the nodose and jugular ganglia [17]. Occasional reports have suggested that paraganglia may be found in other sites such as the gallbladder [18,19], where they may be associated with tiny branches of the vagus nerve. Other locations, such as orbit and extremities, are more difficult to explain in embryological terms. As with sympathetic paraganglia, the amount of parasympathetic paraganglionic tissue at individual sites may change with age.

CATECHOLAMINE AND PEPTIDE PRODUCTION All paraganglia have the capacity to synthesize and secrete catecholamines. The biosynthetic pathway is shown in Fig. 2. Tyrosine comes from dietary sources or from the hepatic conversion of phenylalanine. It is converted to dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase (TH), and decarboxylated to produce dopamine. The enzymes involved in these steps are localized in the cytosolic compartment of the cell. Dopamine is transported into neurosecretory granules for the synthesis of norepinephrine (NE). NE is taken back into the cytosol for conversion to epinephrine, which is transported back into secretory granules for storage. The amounts of catecholamines produced and the balance of the individual products vary with the particular paraganglion. Sympathetic paraganglia on average contain higher concentrations than parasympathetic. The main catecholamine produced by the adrenal medulla is epinephrine. NE predominates in the extraadrenal sympathetic paraganglia as glucocorticoids are required to induce expression of phenylethanolamine *N*-methyltransferase (PNMT), the enzyme that catalyzes the conversion of NE to epinephrine [20,21]. Parasympathetic paraganglia produce very little epinephrine, but may contain high levels of dopamine.

Catecholamines are stored in neurosecretory granules with a variety of proteins. Chromogranin A, an acidic glycoprotein, is a major component [22]. The other members of the family, chromogranin B [23] and secretogranin I, are also stored but at

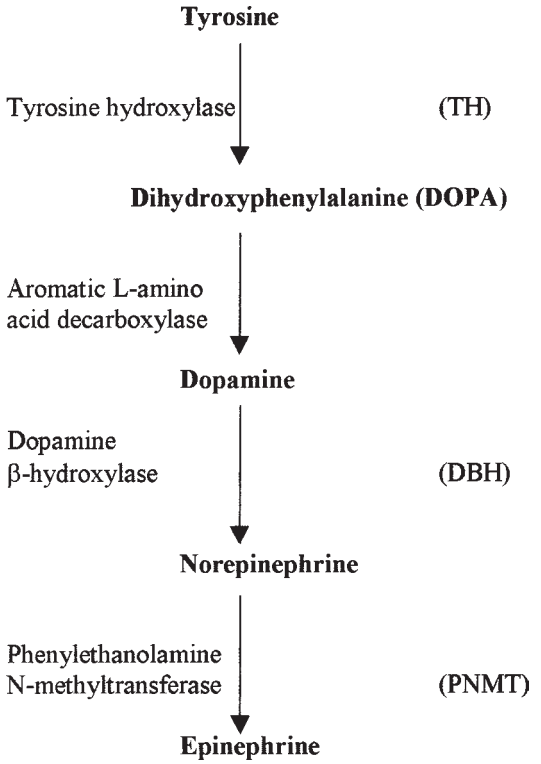

Figure 2 Pathway of biosynthesis of catecholamines. The catecholamines produced vary in each paraganglion. For example, glucorticoids induce the expression of PNMT, and, thus epinephrine is the main product of the adrenal medulla.

much lower concentrations. A range of signaling peptides are also produced as shown in Table 1 [24–36]. The role of many of these is unknown, but neuropeptide Y (NPY) appears to have an autocrine role in catecholamine release in the adrenal medulla [37], as do vasoactive intestinal peptide (VIP) [32,33] and endothelin [34]. Endothelins may also be involved in the response of the carotid body to hypoxia [38]. Growth factors such as fibroblast growth factors (FGFs) and transforming growth factor-β may have a role in regulating proliferation and the maintenance of innervation in the adrenal medulla [39], and there is experimental evidence that basic FGF (bFGF) may stimulate proliferation in the carotid body [40]. The adrenal medulla appears to express somatostatin receptor 2 [41].

HISTOLOGY AND IMMUNOHISTOCHEMISTRY In both types of paraganglion, nests of neuroendocrine cells are surrounded by sustentacular cells, with a variable fibrovascular component. The pattern is more pronounced in parasympathetic paraganglia, giving rise to the characteristic "zellballen." The neuroendocrine cells are polyhedral with abundant cytoplasm and small, often eccentric, nuclei with coarse clumped chromatin and a single nucleolus. In the adrenal medulla and other sympathetic paraganglia they are often referred to as chromaffin cells because of their positivity in the chromaffin reaction discussed above. An alternative name in the adrenal medulla is pheochromocytes. In parasympathetic paraganglia they are called glomus, type 1, or chief cells. Hyaline eosinophilic globules, up to 20 μm in diameter, may be found in pheochromocytes of the adrenal medulla [42]. These are periodic acid-Schiff (PAS) positive and diastase resistant. Some cellular and nuclear

Table 1
Peptides Produced in Paraganglia

Peptide	*Reference*
Galanin	[24]
Enkephalins	[25]
Neuropeptide Y	[26,27]
Peptides derived from proopiomelanocortin	[28]
Parathyroid hormonelike peptide	[29]
Pancreatic polypeptide	[30]
Cerebellin	[31]
Vasoactive intestinal peptide	[32,33]
Endothelin	[34]
Adrenomedullin and related peptides	[35]
Survivin	[36]

pleomorphism may develop with increasing age. Ultrastructural analysis demonstrates the characteristic membrane-bound neurosecretory granules of varying shapes and sizes [43]. The cells may also contain small synapticlike vesicles that tend to sit close to the cell membrane [44]. Sustentacular cells (also known as satellite or type 2 cells) have a dendritic shape, but are not easily seen on hematoxylin and eosin (H&E) staining. They may be localized by immunostaining for S100 protein [45]. They sit mainly on the periphery of the cell nests and extend processes around the neuroendocrine cells. Some are also immunopositive for glial fibrillary acidic protein (GFAP) [46]. Ganglion cells and nerve fibers are commonly found in the adrenal medulla, where most of the neuroendocrine cells are innervated [47]. However, innervation of other paraganglia is less pronounced. All paraganglia have a prominent vascular network and the neuroendocrine cells often sit close to the capillaries.

The neuroendocrine cells stain positively for the commonly used general neuroendocrine markers. These include the cytosolic marker PGP 9.5, a ubiquitin hydrolase [48]; the neurosecretory granule protein chromogranin A [45]; and synaptophysin, a synaptic vesicle protein [49]. Neuron-specific enolase (NSE) is not recommended because of lack of specificity. The cells may also stain for a range of other proteins associated with neuroendocrine activity, including synaptic vesicle proteins SNAP-25 [50] and SV2 [51]. They will also stain positively for enzymes in the catecholamine biosynthetic pathway [49] and for enzymes involved in processing peptide signaling molecules, including proconvertases [52] and peptidylglycine α-amidating monooxygenase (PAM) [53].

Adrenal medullary nerves have been shown to be immunopositive for substance P, dynorphin, and cholecystokinin [54]. Nerves in paraganglia in the bladder have been reported positive for VIP and calcitonin gene related peptide (CGRP) [55] and the carotid body contains VIP, CGRP, NPY, and substance P immunoreactive fibers [56]. Nitric oxide may also to be important in the neural regulation of function of both types of paraganglia [57,58].

INCIDENCE OF PARAGANGLIOMAS

The true overall incidence of paragangliomas is unknown, but a recent conservative estimate of approx 1/300,000 has been made [59]. Population studies on pheochromocytoma report an annual incidence of between 0.4 [60] and 9.5 [61] per 1,000,000.

Based on surgical experience in the Netherlands, an incidence of 1/1,000,000 has been calculated for paragangliomas of the head and neck [62]. The relative distribution of sites in a series of 236 cases of paraganglioma [63] has allowed a calculation that paragangliomas at other sites may have an incidence of 0.45/1,000,000 [59].

Most paragangliomas are sporadic, but approx 10% occur in familial settings. Pheochromocytomas are reported in 30–70% of patients with multiple endocrine neoplasia (MEN) syndromes, types 2A and 2B [64] but extraadrenal tumors are uncommon. They coexist with medullary carcinoma of thyroid in MEN 2A, with the addition of mucocutaneous neuromas in MEN 2B. They are also found in neurofibromatosis type 1 (NF1), occurring in 0.1–5.7% of patients [65]. There is also an association with von Hippel–Lindau syndrome [66,67] and Sturge–Weber syndrome [68]. Familial cases of pheochromocytoma without other features have also been described [69,70]. The proportion of parasympathetic paragangliomas with familial links has been variably reported as 9.5% in a US series [71] and 50% in a study from the Netherlands [72].

In Carney's triad, paragangliomas of either or both types are found in association with pulmonary chondroma and gastric epithelioid leiomyosarcoma [73,74]. The association of a pheochromocytoma with chondrosarcoma has been proposed as a variant of this syndrome [75]. A new autosomal dominant syndrome has recently been described in which pheochromocytoma coexists with gastric stromal sarcoma [76].

SYMPATHETIC PARAGANGLIOMAS

GENERAL FEATURES These occur at all ages, but are commonest in the fourth and fifth decades. About 90% occur in adults and 10% in children. Ninety percent of tumors in adults are intraadrenal. In children, they are divided equally between intra- and extra-adrenal tumors [77]. About half of extraadrenal tumors arise in the organs of Zuckerkandl and the rest are mainly in the retroperitoneum. Other sites include the bladder [78,79] with occasional lesions in kidney, urethra, and prostate or in the chest or neck [68]. In general, there is an equal sex distribution, but males are more often affected in children, and patients with thoracic tumors [64].

NORMAL ADRENAL MEDULLA The normal adult human adrenal gland comprises the outer cortex and the central medulla. It weighs about 4 g in cases of sudden death [80] and 6 g at hospital autopsy, reflecting the hypertrophy of the cortex associated with stimulation by adrenocorticotrophin (ACTH) in the stress of terminal illness [81]. The medulla accounts for about 10% of the normal gland [80,82] and is present only in the head and body, with minor extension into the alae. There is normally no medullary tissue in the tail. A normal range for adrenal medullary weight has been calculated as 0.47 ± 0.15 g [83]. There is irregularity of the cortical/medullary junction in the human adrenal gland and intermingling of cortical and medullary cells [84], supporting the hypothesis that each influences the function of the other [84–87].

PHEOCHROMOCYTOMA Pheochromocytoma is usually a sporadic tumor, with only 5% of cases associated with familial syndromes. Sporadic lesions are generally solitary, but about 5% of patients have bilateral tumors. In contrast, in familial disease about 50% are bilateral. Thus, the finding of more than one tumor in an individual indicates the need for a detailed family history. They may coexist with extraadrenal paragangliomas in a small minority of cases [88]. Clinically, patients may present with paroxysmal or sustained hypertension and the majority have severe headaches, particularly during episodes of hypertension. Palpitation, tachycardia, tremor, and other signs of catecholamine excess may also be present. A minority of tumors do not give rise to such symptoms, possibly because enzymes of the catecholamine biosynthetic pathway are not expressed. Alternatively, this may be due metabolism of catecholamines to inactive metabolites by the tumor cells.

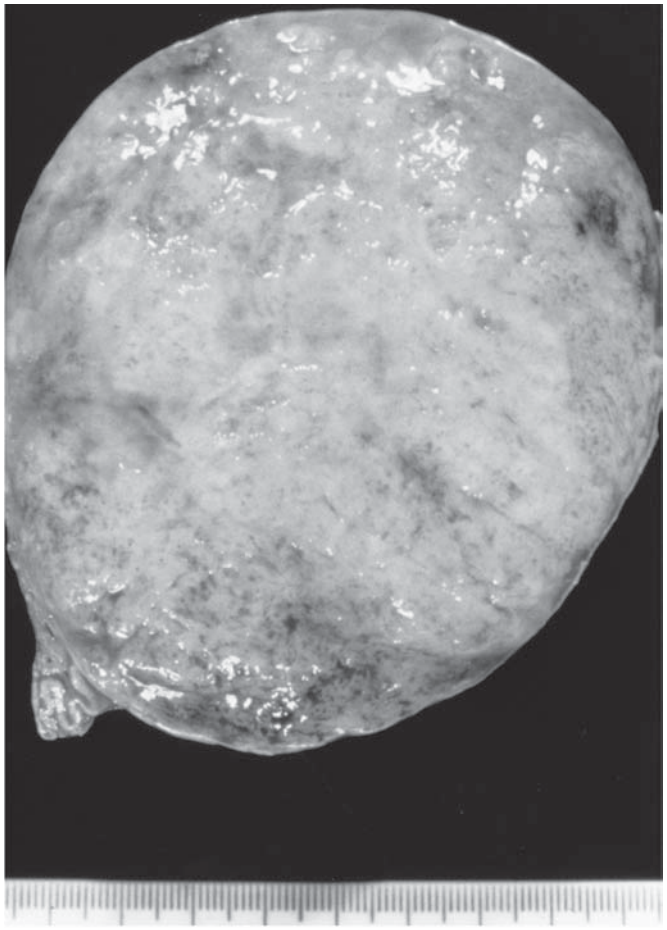

Figure 3 A pheochromocytoma in cross section. The lesion was gray/white, with mottling in the lower part related to hemorrhage. The adrenal gland can be seen at the **bottom left**. The scale is in centimeters.

In the past, a large number of pheochromocytomas were found only at autopsy [89,90] but an increasing awareness has led to a greater proportion being diagnosed in life [91]. A number may still have a primary diagnosis at autopsy, a recent survey suggesting that they may account for 0.05% of coroners' autopsies [92]. Undiagnosed lesions now also account for 1.5–18% of adrenal "incidentalomas," picked up when the abdomen is scanned for the investigation of other intraabdominal disease [93–97].

The lesions are usually confined to the adrenal gland (Fig. 3), and may appear to be encapsulated. The normal gland may be

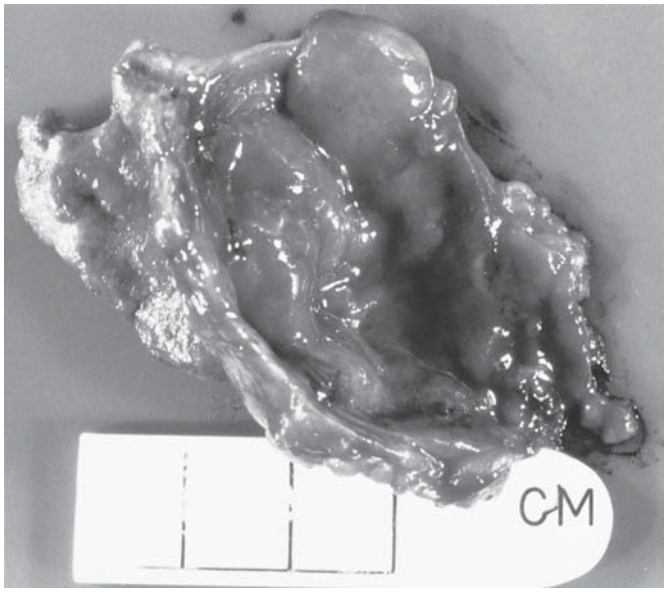

Figure 4 Pheochromocytoma showing cystic degeneration.

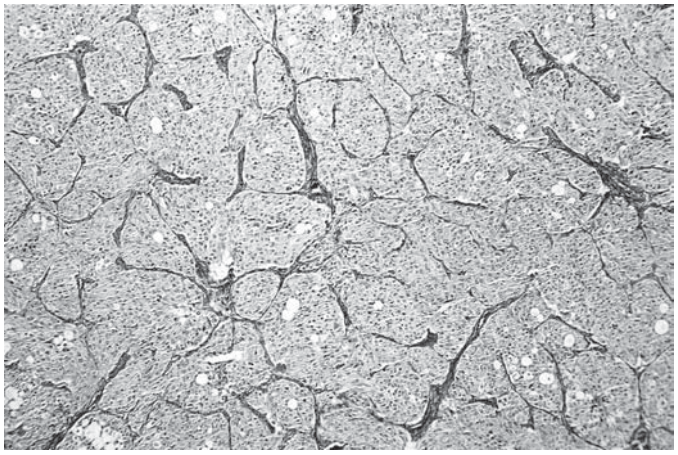

Figure 6 Pheochromocytoma showing a marked alveolar pattern. (Color illustration appears in insert following p. 148.)

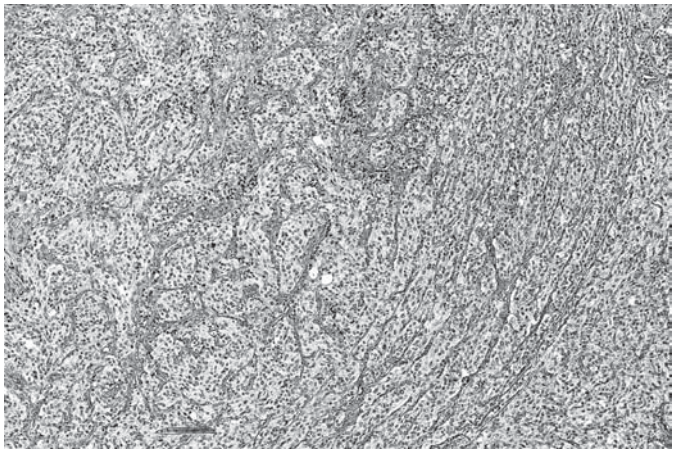

Figure 5 Pheochromocytoma showing mixed alveolar (**left**) and trabecular (**right**) architecture. (Color illustration appears in insert following p. 148.)

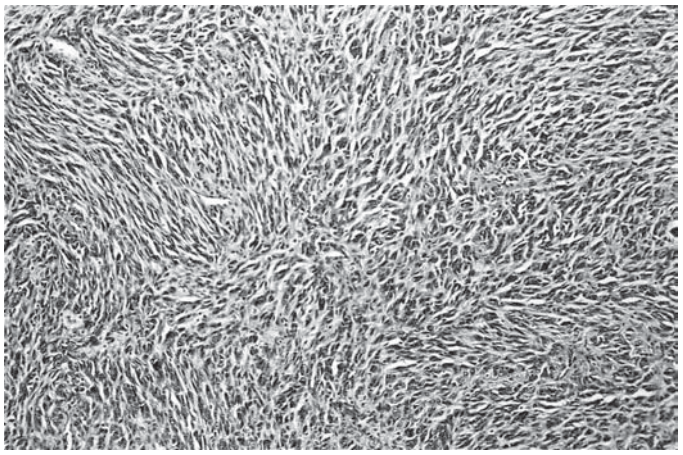

Figure 7 Paraganglioma with a spindle cell morphology. The tumor was immunopositive for general neuroendocrine markers. (Color illustration appears in insert following p. 148.)

obvious, or may be attenuated over the surface of large tumors. Most are between 3 and 5 cm in diameter [98] but the size may range from 1 to >10 cm. Pheochromocytomas weighing <5 g to >3500 g have been reported, the average in people with hypertension being 100 g [99]. The cut surface is gray/white in color and may darken on exposure to air. Focal hemorrhage and central degenerative change are not uncommon. In a few cases, there may be cystic change (Fig. 4). Some tumors show calcification. Malignant lesions may show evidence of invasion of surrounding structures, such as kidney or liver. Rarely, there is extension into the inferior vena cava.

Microscopic examination most commonly shows a mixed alveolar and trabecular arrangement (Fig. 5). In some tumors, one or the other of these patterns predominates (Fig. 6). In approx 2% of cases a spindle component may be found, but only rarely predominates (Fig. 7). Focally, areas with more diffuse or solid architecture may be identified. The border with the adjacent cortex may be irregular. In general, the cells resemble pheochromocytes, but in some tumors cellular and nuclear pleomorphism is pronounced (Fig. 8) and nuclear pseudoinclusions are seen [100], while others are composed of small cells [101]. Intracellular hyaline globules may be a feature (Fig. 9). Variable amounts of melaninlike pigment may occasionally be seen (Fig. 10) [102,103]. This is neuromelanin, derived from a combination of breakdown products of tyrosine and lipofuscin. It stains positively with the Fontana stain for melanin, but usually requires prolonged bleaching as used on brain sections to abolish staining. Occasional mitotic figures are present, an average of 1 per 30 high-power fields reported in clinically benign lesions in one study [104]. Cells resembling ganglion cells and neuroblasts are occasionally seen. Sometimes the cells may undergo "lipid degeneration," assuming a clear cell appearance, which may mimic an adrenal cortical tumor (Fig. 11) [105,106]. Oncocytic tumors have been described [107]. Stromal sclerosis may be marked and amyloid has been demonstrated [108]. The vascular component is often prominent.

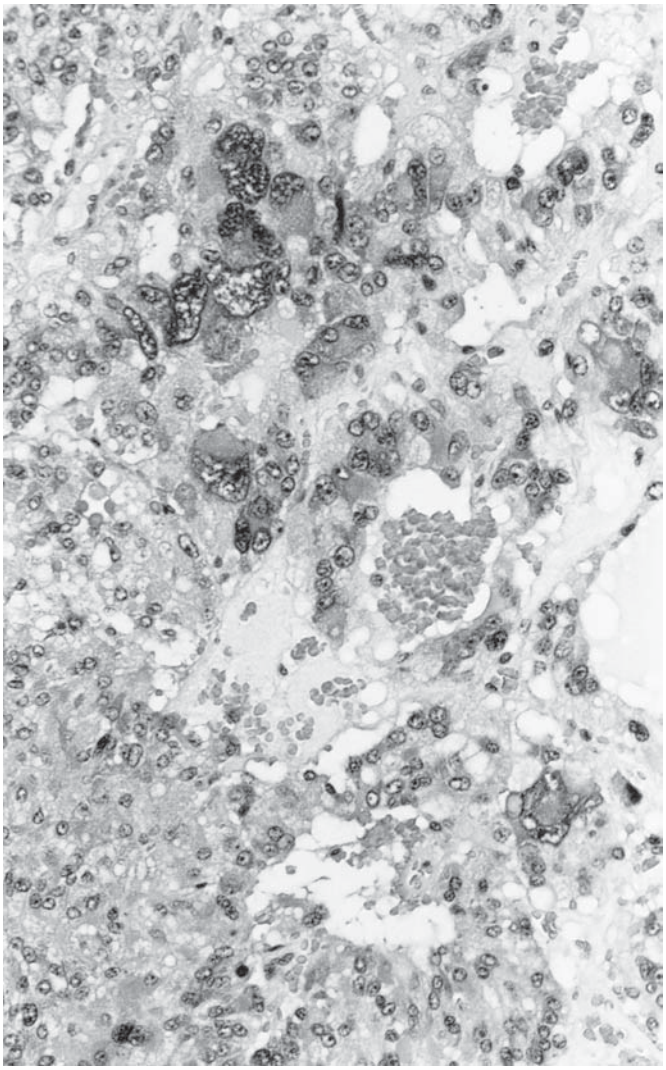

Figure 8 Marked nuclear pleomorphism is present in this pheochromocytoma.

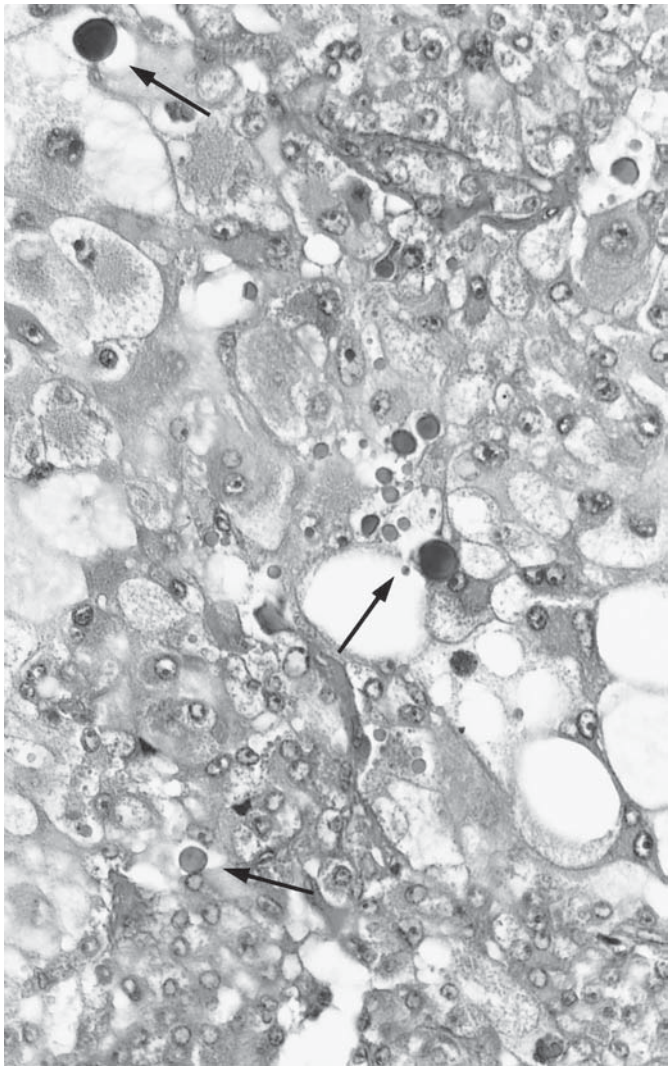

Figure 9 Intracytoplasmic hyaline globules are present (*arrows*).

The lynchpin of specific diagnosis is immunohistochemistry, and histochemical techniques such as the chromaffin reaction and silver stains should be abandoned. As in other neuroendocrine tumors, there is immunopositivity for the general neuroendocrine markers synaptophysin (Fig. 12), PGP 9.5, and chromogranin A (Fig. 13). They may also express neurofilament [109,110]. Immunostaining for S100 protein will demonstrate sustentacular cells [45] (Fig. 14), although positive staining of tumor cells in some cases can make the stain difficult to interpret. As in the normal gland, a subpopulation of sustentacular cells may show positivity for GFAP. A number of the peptides produced by the normal medulla are also expressed in tumors [111], although these are not normally documented as a diagnostic procedure. The exception would be the demonstration of hormones such as ACTH in cases of ectopic hormone secretion associated with a clinical syndrome [112,113]. Specific identification of paraganglioma can be made by immunopositivity for the enzymes involved in the synthesis of catecholamines.

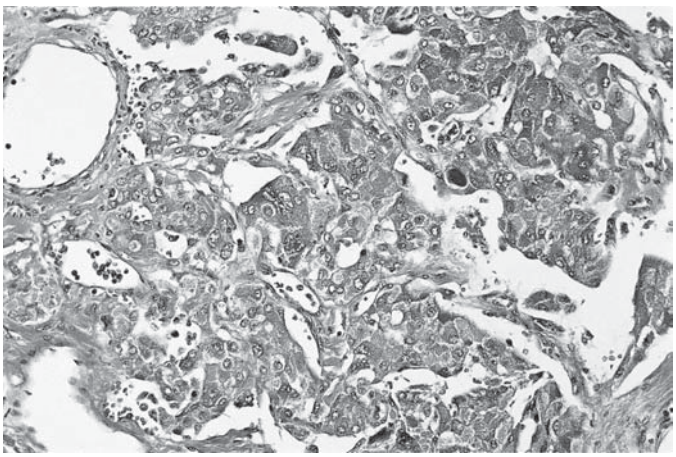

Figure 10 Significant amounts of brown pigment are present in the cytoplasm of the tumor cells in this pheochromocytoma. This gave a positive reaction for melanin. (Color illustration appears in insert following p. 148.)

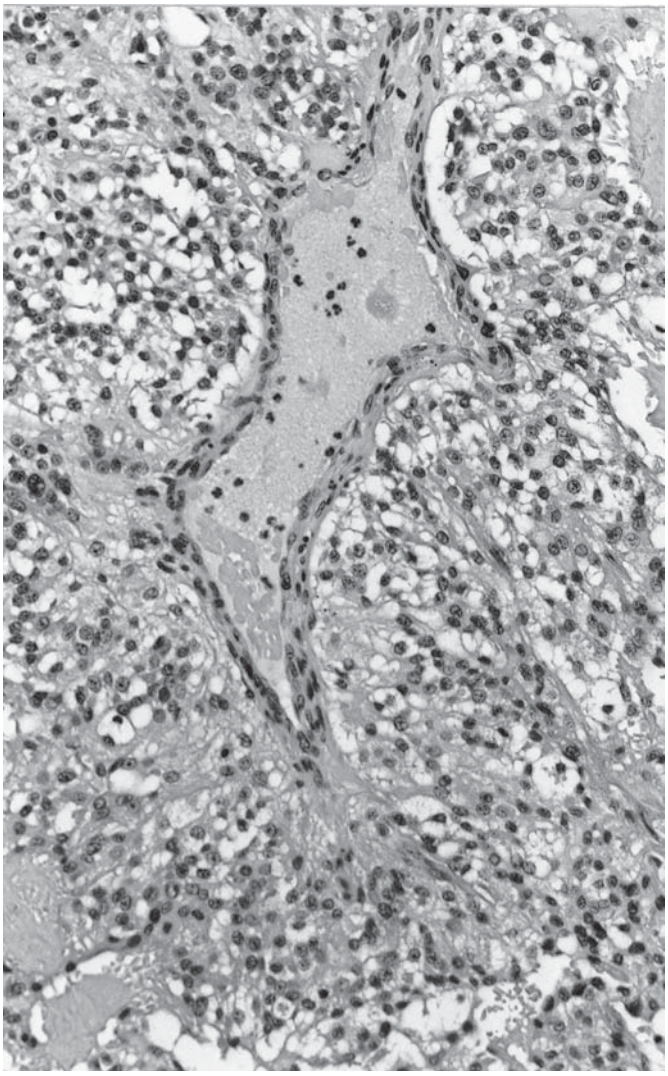

Figure 11 Clearing of the cytoplasm in this tumor produces an appearance similar to an adrenal cortical tumor. Immunostaining for neuroendocrine markers was positive.

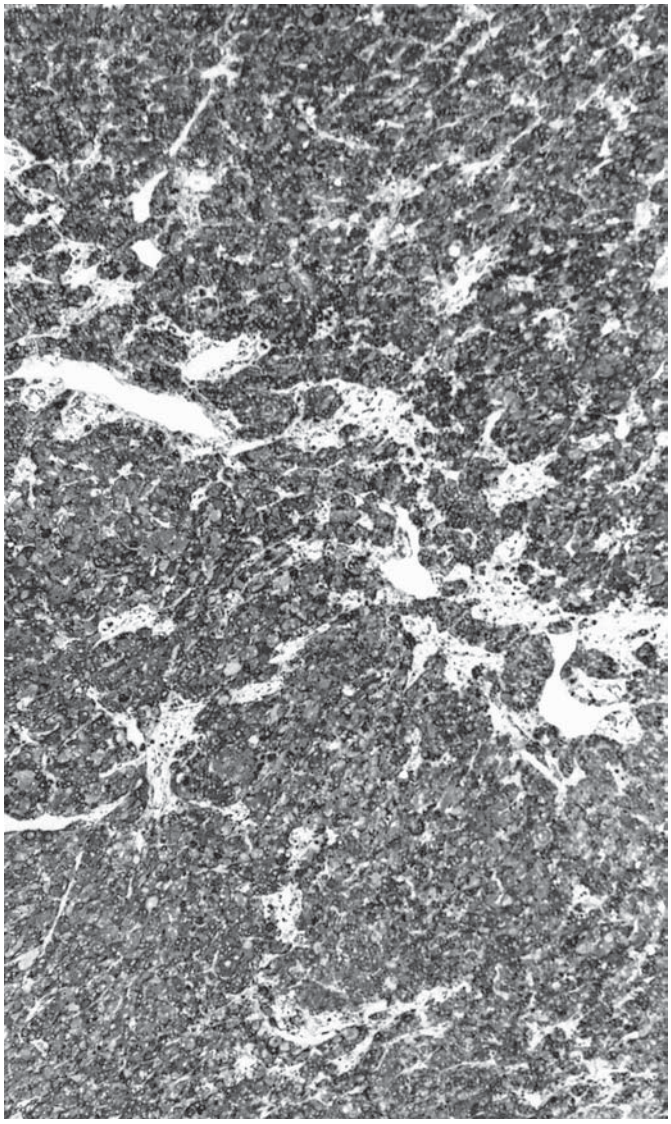

Figure 12 Pheochromocytoma showing generalised immunopositivity for synaptophysin.

COMPOSITE PHEOCHROMOCYTOMA Because the embryologic precursor cells of paraganglia have the potential to give rise to neuroendocrine cells, nerves, and ganglion cells, a minor component of these elements is a recognized feature of pheochromocytoma. Sometimes this is so prominent as to warrant a diagnosis of composite pheochromocytoma, constituting 3% of cases in one series [104]. These mixed tumors have been reported to occur more commonly in neurofibromatosis [114]. The second component may resemble neuroblastoma, ganglioneuroblastoma, ganglioneuroma, or, rarely, malignant schwannoma [115]. The behavior of such tumors is difficult to predict. The presence of a histologically malignant component may be associated with metastasis and poor outcome, but does not always infer a poorer prognosis [68].

ADRENAL MEDULLARY HYPERPLASIA Adrenal medullary hyperplasia (AMH) is an increase in the number of chromaffin cells within the adrenal gland. It can be diagnosed with certainty only by morphometric analysis [116,117], but in general diagnostic practice is recognized as an extension of the medullary tissue into the tail or alae of the gland where it is normally absent or sparse. Planimetric analysis has shown that the normal corticomedullary ratio is about 10:1 [118]. However, when assessing the relative proportions of cortex and medulla, it has to be remembered that a reduction in the volume of the cortex may give a relatively higher proportion of medulla. In these circumstances, it is possible to calculate the absolute medullary weight [83]. Hyperplasia may be diffuse or nodular or a combination of both. The distinction between nodular hyperplasia and pheochromocytoma can be difficult, and an arbitrary cutoff point of 1 cm diameter has been proposed [119]. The demonstration of clonal lesions in both of these groups suggests that some lesions smaller than 1 cm may indeed be neoplastic rather than hyperplastic [120].

AMH is a well-recognized precursor to pheochromocytoma in MEN 2 [83,119] (Fig. 15). A recent study suggests that it is not a precursor in von Hippel–Lindau disease [121]. There are

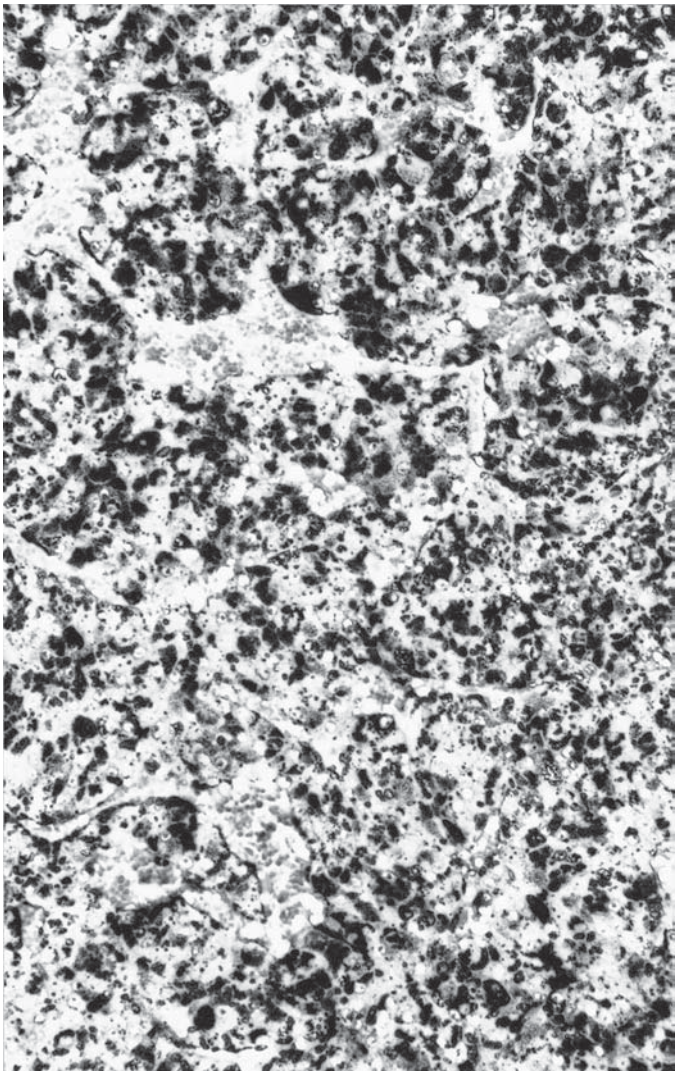

Figure 13 Pheochromocytoma showing granular staining for chromogranin A.

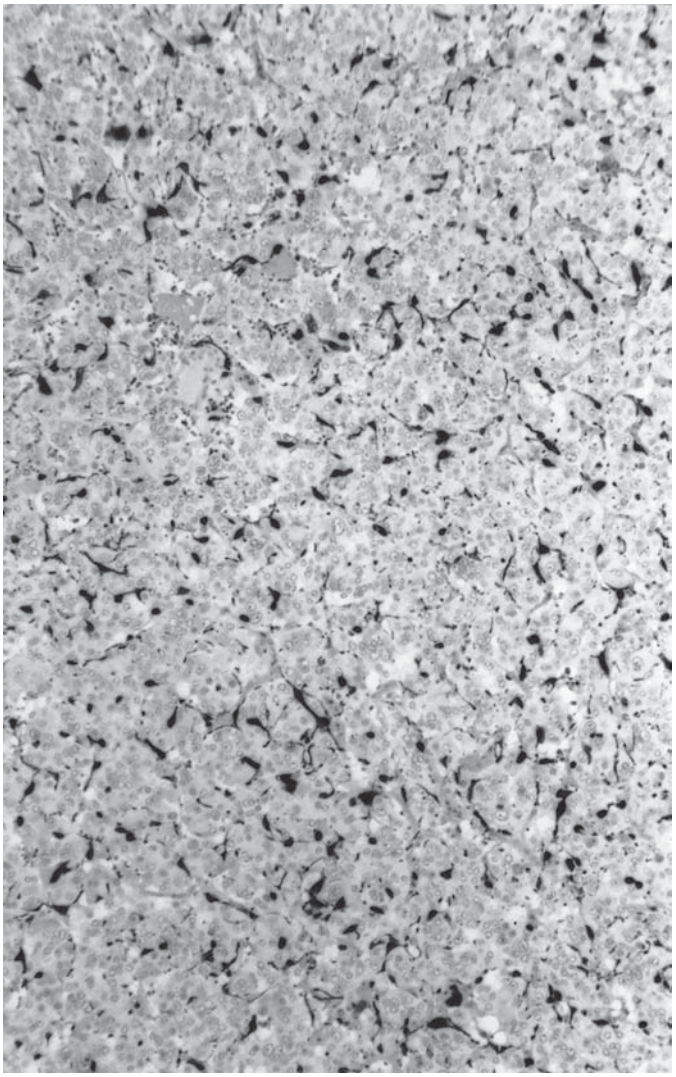

Figure 14 Immunostaining for S-100 protein demonstrates dendritic sustentacular cells in this paraganglioma.

no published data on neurofibromatosis. Sporadic bilateral or unilateral hyperplasia has been documented as a cause of hypertension [116,122]. Whether this may be a precursor of pheochromocytoma is not known.

AMH has also been described occasionally in sudden infant death syndrome [123], in Beckwith–Wiedemann syndrome, and in association with adrenocortical adenoma [124,125]. These may represent chance associations rather than causal events.

EXTRAADRENAL SYMPATHETIC PARAGANGLIOMAS The relative distribution of extraadrenal paragangliomas is shown in Table 2. Between 25% and 75% are functional [126] and 8% [127] to 50% [128] metastasize. Histologically, they show the typical features. They will usually show positivity for the early enzymes in the catecholamine pathway, TH, and DBH, but almost never for PNMT.

Urinary bladder tumors are worthy of mention. They affect males and females equally, and occur usually in the trigone, but may be in the dome or lateral walls. The majority of patients have the clinical triad of paroxysmal hypertension, gross intermittent hematuria, and intermittent episodes of symptoms related to catecholamine release, such as headache, palpitation, and anxiety. These may be triggered by micturition. The tumors are usually small, ranging from 0.3 to 5.5 cm [78], and may project into the bladder lumen. Many interdigitate with muscle bundles, but this does not indicate malignant potential.

Intrathoracic paravertebral paragangliomas lie close to the sympathetic axis and are most commonly found in the midthoracic region [126]. About 70% arise in males and half are functional. Cervical lesions are extremely rare and their behavior is not clear.

Paraganglioma of the cauda equina is a rare tumor, usually intradural and involving the filum terminale [129]. These tumors are consistently immunopositive for cytokeratin, and occasional lesions at other sites may show positivity [79,130–132].

DIAGNOSIS OF MALIGNANCY Malignancy can be diagnosed with certainty only when there is extensive local invasion or metastasis (Fig. 16). Only about 5% of pheochromocytomas metastasize, but up to 10% show local recurrence [99]. Thirty percent of extraadrenal intraabdominal tumors are malignant, while 5% [78] to 13.8% [133] of bladder lesions have metas-

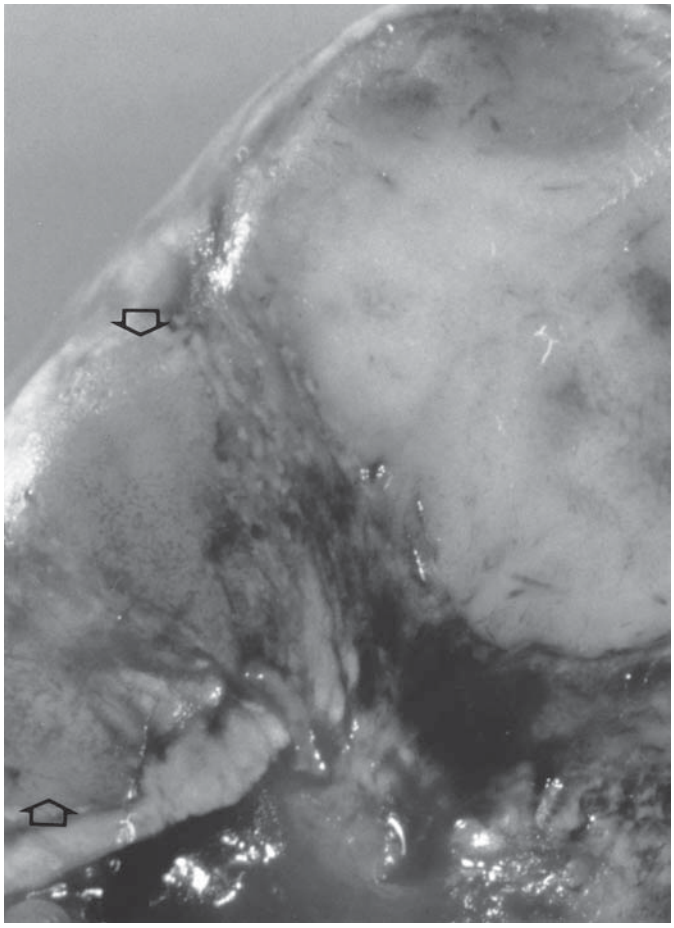

Figure 15 Adrenal gland from a patient with multiple endocrine neoplasia, type 2. There is an intraadrenal pheochromocytoma on the **right**. On the **left**, *between the arrows* medullary hyperplasia is present in the ala of the gland.

Table 2
Distribution of Extraadrenal Sympathetic Paragangliomas [219]

Site	*Percentage of cases*
Head and neck	3
Thorax	10
Superior paraaortic	46
Inferior paraaortic	29
Bladder	10
Pelvis	2

tasized to local lymph nodes or distant sites. There are no reliable criteria for predicting malignant potential, and therefore none of these lesions should at present be defined as benign. Published data on a large series of sympathoadrenal paragangliomas found only four features that had a statistical correlation with malignancy: extraadrenal location, coarse nodularity of the primary tumor, confluent tumor necrosis, and absence of hyaline globules [104]. Malignant tumors are heavier than benign tumors (average of 383 g compared with 73 g), have a higher mitotic count (average of 3 per 30 high-power fields compared with 1), and have extensive local or vascular invasion. Small cell morphology is more commonly seen in malignant tumors [101]. There has been a recent attempt to devise a numerical system for assessing behavior of pheochromocytoma, based on assigning a value to histological features including vascular invasion and invasion of surrounding fat (Fig. 17) [134]. The figures are summed to indicate the potential for metastasis. While this did separate groups already known to have had different behavior, it needs to be tested prospectively on a larger series of cases. Some groups have suggested that the range of peptides expressed differs in benign and malignant tumors [135–137], but the data are not consistent and this is not of importance in diagnostic practice. Others report the absence of sustentacular cells in malignant tumors [46,138] but the author has seen a metastasizing tumor containing S100-positive sustentacular cells. In contrast, tumors with no evidence of spread can have foci where these cells are absent. Interpretation of S100 staining may be difficult if the tumor cells are immunopositive.

There has been recent interest in attempting to correlate proliferation index, as identified by MIB-1 immunostaining, with behavior (Fig. 18). Using a cutoff point of 2.5% [139] or 3% [140] gave 100% specificity, but only 50% sensitivity, in identifying malignant tumors. Thus, it should be noted that not all malignant pheochromocytomas have a high level of proliferation. The bulk of evidence now suggests that ploidy analysis is not of diagnostic value in individual lesions [141–143]. Newer approaches include the examination of various oncogenes and tumor suppressor genes. The data are still experimental, the number of cases studied to date is relatively small, and some studies have conflicting results.

PARASYMPATHETIC PARAGANGLIOMAS

GENERAL FEATURES Jugulotympanic paragangliomas have been reported as the most common (57–81%), with carotid body (4–13%), vagal (4–13%), and aortic (4–10%) comprising most of the rest [144]. However, in some series carotid body tumors predominate [145,146]. Sporadic tumors occur more commonly in females but familial tumors have an equal sex distribution. The exact ratio may vary at different sites [146, 147]. Only about 1% produce symptoms of catecholamine excess [144], which may reflect the fact that physiologically they produce only small quantities of catecholamines.

Carotid body tumors present most commonly in the fifth decade [145,148], but can occur at any age. There is a slight female predominance. They are bilateral in 3–8% of sporadic cases and 38% of familial cases [144,149]. The majority show some adherence to the adventitia of the carotid artery and some completely surround the bifurcation [150]. A chronic inflammatory infiltrate is not uncommon.

The incidence of paraganglioma of the carotid body is 10 times greater in people living at high altitude than at sea level, and there is a female-to-male ratio of 6:1 [151]. This may be related to hyperplasia induced at altitude [152], most probably in response to the hypoxic stimulus. Hypertrophy and hyperplasia also occur in people with chronic obstructive airways disease [153–156] and in patients with cystic fibrosis and cyanotic heart disease [157]. Increased proliferation has been demonstrated in the carotid bodies of rats subjected to hypoxia [158].

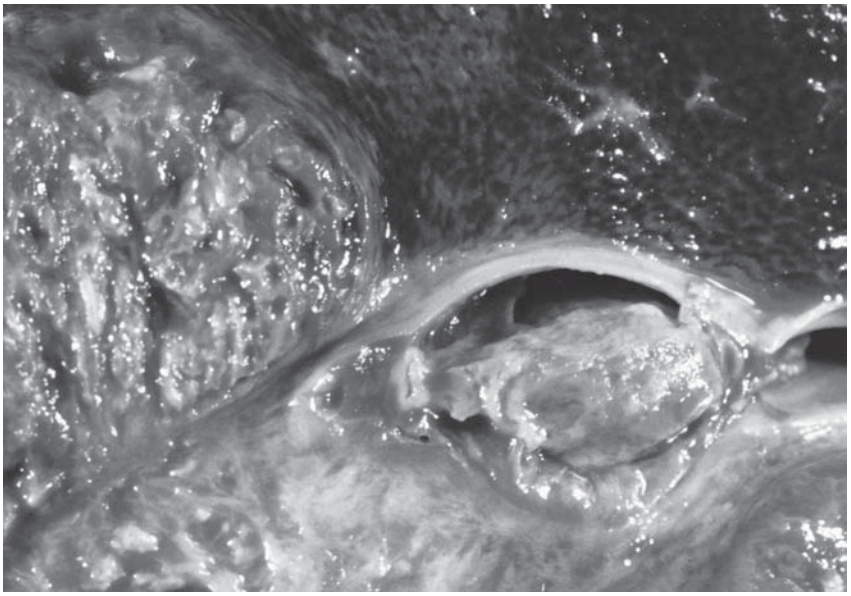

Figure 16 Metastatic pheochromocytoma, invading liver and portal vein.

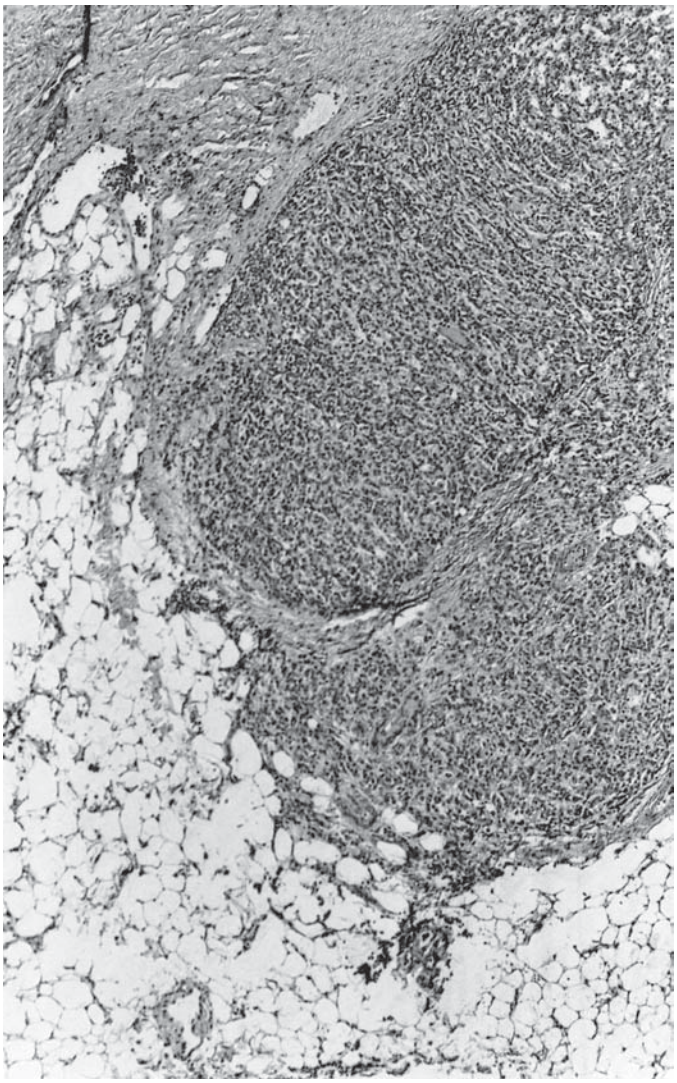

Figure 17 Pheochromocytoma invading periadrenal fat.

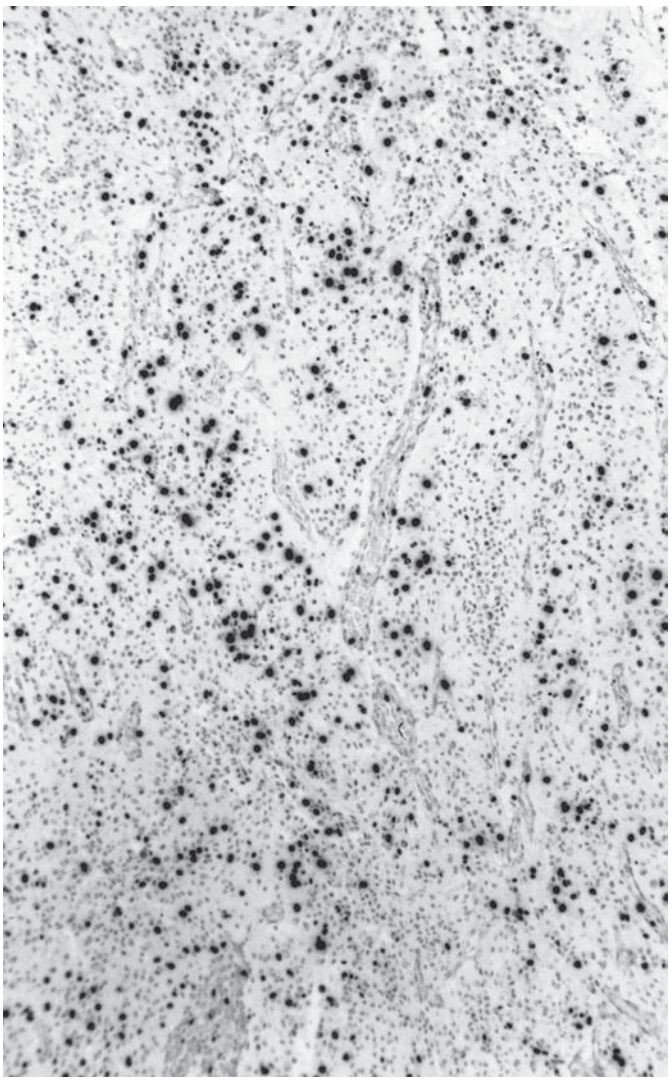

Figure 18 Extraadrenal paraganglioma immunostained with MIB-1. This tumor had a very high MIB-1 index (>10%).

Jugulotympanic paragangliomas are slow growing lesions that usually present in the fifth and sixth decades, with a female-to-male ratio of 6:1 [159]. Glomus jugulare tumors produce cranial nerve palsies, while glomus tympanicum tumors produce tinnitus and hearing loss. Some may mimic meningioma, if they grow intracranially. They are often more vascular than other head and neck paragangliomas, and may show sclerosis and calcification.

Vagal paragangliomas show no age-related peak, but are more common in women. Thirty percent of patients have cranial nerve involvement. However, nests of tumor cells may lie in the nerve fibers, without signifying invasion. Laryngeal lesions present in the fifth decade, usually as a submucosal mass with hoarseness.

Aorticopulmonary and pulmonary lesions arise around the aortic arch or within the heart (usually atria) [160,161] or pericardium [162]. Pulmonary lesions may lie within the lung or near the pulmonary artery [68]. About 20% of these lesions are associated with paragangliomas at other sites [144,161]. The origin of paragangliomas reported at sites where paraganglia are not recognized in the human, such as orbit, parotid, face, and external ear, is unclear.

DIAGNOSIS OF MALIGNANCY As with sympathetic paragangliomas, malignancy can be diagnosed only on the basis of metastasis or extensive local invasion. The published rates of malignancy are between 5% and 15%, but vary with site. The reported incidence of malignancy in carotid body tumors is 6.4–23% [68], with a recent long-term study suggesting the figure is approx 10% [163]. Five percent of jugulotympanic [164] and 25% of laryngeal paragangliomas are malignant. Metastases in the majority of cases are confined to local lymph nodes [165], but distant spread to liver, lung, or bone is recorded. There have been few studies aimed at identifying features associated with malignancy. High mitotic activity, extensive vascular invasion, and central necrosis of cell nests have been documented [68].

DIFFERENTIAL DIAGNOSIS

Most paragangliomas are easy to diagnose, because of their site and their characteristic architectural patterns, but a differential diagnosis may have to be considered in a small proportion of cases. This will either be distinction from other neuroendocrine tumors in more typical cases or from tumors of different histogenesis when the morphology is unusual. Immunohistochemistry usually plays an important role in making the diagnosis.

In the neck, they may need to be distinguished from medullary carcinoma of thyroid. This can usually be done on the basis of widespread positivity for calcitonin and carcinoembryonic antigen (CEA) [166] and thyroid transcription factor 1 (TTF1) [167] in medullary carcinoma. Sustentacularlike cells have been reported in medullary carcinoma [168], so S100 staining is not useful. Hyalinizing trabecular tumors of thyroid are characterized by immunopositivity for cytokeratin [169,170], so negative staining can rule this out as a differential diagnosis. However, as some paragangliomas in this region express cytokeratin [131, 171], positive staining is not helpful in the distinction. Hyalinizing trabecular tumor does not express neurofilament. In the larynx, they should not be confused with atypical carcinoid tumors. These will stain positively with cytokeratin and CEA [172].

In an intraabdominal location, cytokeratin staining alongside a panel of antibodies to gut and pancreatic hormones should permit differentiation from enteropancreatic endocrine tumors. However, the hormonal staining needs to be interpreted with some caution as a number of these peptides, including glucagon, gastrin, somatostatin, pancreatic polypeptide, and VIP, have been reported in paragangliomas [135].

Spindle cell lesions may require distinction from soft tissue tumors, and negative staining for desmin or smooth muscle actin can be useful in ruling out smooth muscle differentiation. Reticulin staining can be useful in differentiation from hemangiopericytoma and glomangioma, the reticulin network surrounding individual cells in those tumors, and cell nests in paragangliomas.

Occasional pheochromocytomas have a lipid-laden appearance, thus resembling adrenal cortical lesions (Fig. 11) [105, 106]. Synaptophysin and PGP 9.5 can be expressed by adrenal tumors [173,174], so chromogranin A is the only useful neuroendocrine marker in making the distinction. The majority of adrenal cortical tumors will stain positively for inhibin-α [175–177] and/or with the A-103 clone antibody to Melan-A [178, 179]. Nuclear staining with antibody D11 is also useful in defining cortical tumors [180,181].

Pigmented paragangliomas may need to be distinguished from metastatic melanoma. Melan-A (either antibody) should identify melanomas [182,183], and is negative in paragangliomas [179]. HMB45 may occasionally stain pheochromocytoma [184,185]. Rarely, melanoma may arise as a primary at this site [186].

MOLECULAR PATHOGENESIS

FAMILIAL PHEOCHROMOCYTOMAS Approximately 6% of patients with pheochromocytoma have evidence of MEN 2, with 95% of these having MEN 2A and 5% MEN 2B. Pheochromocytomas in these patients are usually multicentric. The *MEN2* gene, the *RET* (REarranged during Transfection) proto-oncogene, is located on chromosome 10q11.2 and encodes a transmembrane receptor tyrosine kinase [187]. *RET* is expressed in adrenal medulla and pheochromocytoma [188,189]. It is activated by binding to the glial cell line–derived neurotrophic factor (GDNF) family of ligands, including GDNF, neuturin (NTN), artemin (ART), and persephin (PSP) [190]. *MEN2* is associated with germline mutations in the *RET* gene. Activating missense mutations in the cysteine-rich extracellular region (codons 609, 611, 618, 620, 630, and 634) have been identified in 97% of MEN 2A families [191]. In MEN 2B, an activating mutation of the tyrosine core domain (codon 918: Met $\rightarrow$ Thr) has been shown in 94% of cases. In approximately half of MEN 2B patients, the mutation arises *de novo*.

Familial disease also occurs in von Hippel–Lindau disease [67,192], where it is associated with germline mutations of the *VHL* tumor suppressor gene on chromosome 3p [193]. Pheochromocytomas, and composite tumors, also arise in neurofibromatosis type 1 [65], due to germline mutation in the *NF1* gene, which encodes the protein neurofibrin. Interestingly, mice with targeted disruption of the mouse homologue of *NF1* develop pheochromocytomas [194].

FAMILIAL PARAGANGLIOMAS Paragangliomas may be inherited in a familial pattern without other types of tumors. These are often multiple and the disease has an autosomal dominant transmission with partial penetrance. This syndrome (PGL) has been linked to germline mutations in the genes encoding three subunits of the mitochondrial complex II (succinate dehydrogenase, succinate–ubiquinone oxidoreductase, SDH), a heterotetrameric complex involved in Krebs cycle and the aerobic electron transport chain. *SDHB* (PGL4) maps to chromosome 1p36 [195], *SDHC* (PGL3) to chromosome 1q21 [196], and *SDHD* (PGL1) to chromosome 11q23 [197]. The nature of the mutations predicts loss of function mutant variants [198]. There is subsequent loss of heterozygosity (LOH) of the nonmutated alleles in the tumor [196,197], suggesting that these genes act as tumor suppressors in paraganglia. The mechanisms by which these changes might cause tumorigenesis are unclear. Complex II activity is selectively and completely lost in PGL tumors with *SDHD* mutations [199]. Loss of function of the complex may mimic chronic hypoxic stimulation, which causes proliferation in paraganglia [200]. This theory is supported by the overexpression of hypoxia inducible genes in paraganglioma [199,201].

In PGL1, transmission is only through the father, suggesting genomic imprinting of the maternal allele of the gene involved, although the mechanism is not clear at present [72,202,203]. There is no sex-specific transmission in families with *SDHC* and *SDHB* mutations [195,204,205].

Germline mutations in mitochondrial complex II genes have been demonstrated in some familial pheochromocytomas not associated with the syndromes discussed above. These may coexist with extraadrenal paragangliomas of both sympathetic and parasympathetic types [195,206].

SPORADIC PHEOCHROMOCYTOMAS LOH studies have shown losses on chromosomes 1p, 3p, 11p, 17p, and 22q [207–211]. A comparative genomic hybridization study indicated losses in 1p, 3p, 3q, 6q, 11q, and 17p and gains in 6q and 17q [212]. Deletion of 6q and 17p appeared to correlate with tumor progression.

Patients with pheochromocytoma without a family history of MEN 2, VHL, or NF 1 can have germline mutations in the *RET* or *VHL* genes. In a recent review [59] the prevalence of occult mutations in the *RET* gene is estimated at approx 3.6%, with a higher prevalence of germline mutations in the *VHL* gene (approx 8.5%). The same author has also summarized data on the Complex II genes, calculating a germline mutation rate for *SDHD* of appox 3.5% from a total of 369 cases derived from four studies. For SDHB, the figure is approx 8.0%, based on 291 cases from two series. The suggestion is made that the coexistence of pheochromocytoma with other paragangliomas should raise the possibility of involvement of Complex II genes.

Activation of telomerase has been reported in malignant but not benign pheochromocytomas [213]. Overexpression of *p53* has been reported to be similar in both benign and malignant lesions [214] or more common in malignant lesions [215]. Malignant tumors more commonly express *bcl-2* [215] and show loss of retinoblastoma protein (pRb) [214].

SPORADIC HEAD AND NECK PARAGANGLIOMAS Complex II mutations play a role even in sporadic disease. In a study from the Netherlands, approx 36% of nonfamilial cases have the two founder *SDHD* mutations [216]. A US study showed a lower level, with mutations in SDHD (5%) and SDHB (3%) in nonfamilial disease [204]. Other mechanisms are poorly understood. The proteins c-myc, c-jun, and bcl-2 are expressed in carotid body tumors [217] and overexpression of mdm-2 has recently been reported in paragangliomas [218].

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13 Endocrine Ovaries

Xavier Matias-Guiu, MD, PhD, Juan J. Espinós, MD, and Joaquim Calaf, MD

HISTOLOGY OF THE OVARY

SURFACE EPITHELIUM AND EPITHELIAL INCLUSION CYSTS The ovaries are covered by a single layer of modified peritoneal cells that vary from flat to columnar. The surface epithelium is separated from the underlying stroma by a distinct basement membrane [1].

Epithelial inclusion cysts are considered invaginations of the surface epithelium that have lost any connection with the surface. With advancing age, the frequency of inclusion cysts increases. The epithelium of the cysts may become hyperplastic or undergo metaplasia into different müllerian cell types (ciliated, mucinous, endometrioid, or even transitional). Epithelial inclusion cysts are thought to result from scarring following ovulation. It is widely accepted that they are responsible for the development of benign, borderline, or malignant epithelial–stromal ovarian tumors [2].

STROMA The ovarian cortical stroma is composed of spindle-shaped cells arranged in whorls with a storiform pattern. In women in the reproductive age group, the stroma contains follicles, corpora lutea, and corpora albicantia. Some of the stromal cells may exhibit microscopic features associated with steroid hormone production, such as condensation or luteinization. The ovarian cortical stroma may occasionally contain other elements such as bundles of smooth muscle, decidual cells, fat cells, cortical poorly defined granulomas, and scars.

FOLLICLES AND DERIVATIVES The ovarian stroma contains follicles in different stages of maturation (primordial, primary, secondary, graafian). The number of primordial follicles is estimated to be approx 400,000 at birth.

Primordial follicles are composed of a primary oocyte surrounded by a single layer of inconspicuous granulosa cells (Fig. 1). After gonadotropin stimulation, the oocyte enlarges and the granulosa cells become polygonal (primary follicle).

Follicular maturation produces changes in the morphology of the follicles (secondary follicles). Granulosa cells proliferate and become stratified (preantral follicle), and the surrounding cortical stroma differentiate into both theca interna and externa. At the preantral stage, the enlarging oocyte is surrounded by a eosinophilic, periodic acid-Schiff (PAS)-positive thin layer named "zona pellucida." Further maturation leads to the accumulation of fluid among the granulosa cells (antral follicle). In the mature graafian follicle, the oocyte is covered by few layers of granulosa cells and protrudes into the cavity, in an eccentric position (cumulus oophorus) (Fig. 2).

This cyclical process is the consequence of a complex relationship between gonadotropin hormones (luteinizing hormone [LH] and follicle-stimulating hormone [FSH]) and ovarian markers of follicular development. The decrease of ovarian steroids and inhibins from the corpus luteum of the previous cycle induces an increase in the secretion of both LH and FSH by the pituitary. However, the gonadotroph cell has the ability to modulate the relative amount of both hormones in different stages of the follicular growth. Thus, at the early stage of follicular development FSH is secreted predominantly.

The two cells involved in follicular growth (granulosa and theca) are able to bind both LH and FSH. However, the availability of LH and FSH receptors is higher in theca and granulosa cells, respectively. The subtle interaction between these elements generated the concept of the "two-cell, two-hormone" theory. According to this theory, LH binds to theca cells to induce the secretion of androgens (mainly androstenedione), which are aromatized in granulosa cells to produce estrogens.

Although several follicles start the process of follicular maturation during each menstrual cycle, only the *dominant follicle* completes the development to mature (graafian) follicle. Most follicles undergo atresia. In the absence of ovulation, the basement membrane between granulosa cells and theca cells becomes hyalinized, and the follicle is eventually converted into a corpus fibrosum. The process of selecting the *dominant follicle* among the developing cohort is also regulated through a negative feedback system. The growing follicles produce a progressive increase of serum levels of estradiol and inhibins, which induce a reduction in the pituitary secretion of FSH. As the ability of binding FSH is closely related to the number of granulosa cells in each follicle, the *dominant follicle* faces the shortage in circulating levels with clear advantage. The *dominant follicle* produces estradiol, which induces a negative feedback reaction on FSH secretion and starvation and atresia of the smaller follicles. Androgens also play a role in this process. The variation in the relative levels of LH and FSH stimulates the theca cell

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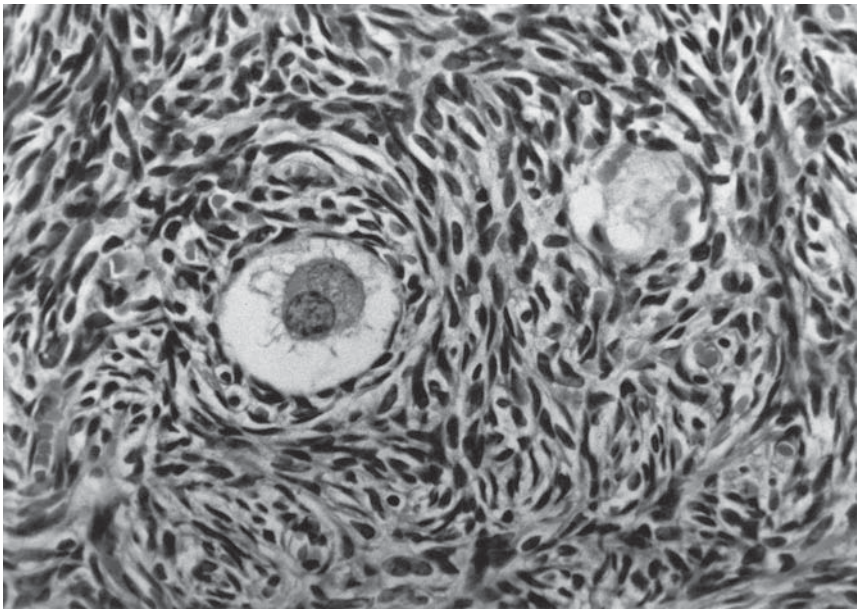

Figure 1 Microscopical appearance of a primordial follicle. The oocyte is surrounded by a thin layer of flat granulosa cells.

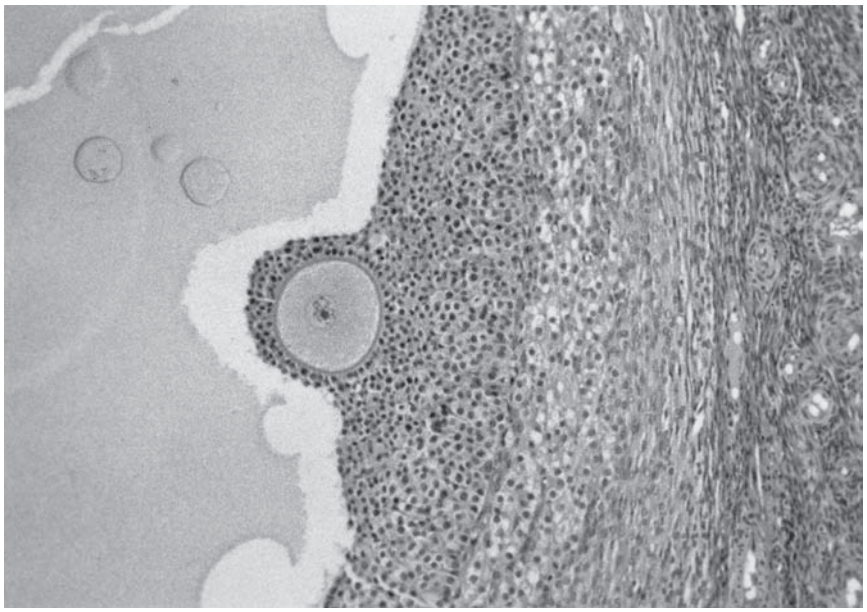

Figure 2 A mature (graafian) follicle. The oocyte is surrounded by a few layers of granulosa cells, protruding into the follicular cavity.

component of the smaller follicles that produce androgens that cannot be aromatized into estrogens by the progressively decreasing granulosa cell population. The imbalance between FSH and LH levels plays a role in the pathogenesis of polycystic ovarian syndrome, as described later.

After ovulation, the disrupted mature follicle becomes a corpus luteum. It is composed of a thick layer of luteinized granulosa cells and an outer, thinner layer of theca–lutein cells. Granulosa cells become large polygonal cells with eosinophilic cytoplasm containing lipid vacuoles. The theca interna forms an irregular and often interrupted layer that originates capillaries that penetrate the granulosa cell layer and reach the central cavity. The central cavity of the corpus luteum becomes lined by an inner fibrous layer composed of fibroblasts that accompany the capillaries and a dense reticulum network.

This structure is maintained by episodic increases of circulating LH. Hormone secretion by the pituitary cell is controlled by the serum levels of both estradiol and progesterone secreted by the corpus luteum. Moreover, both estradiol and progesterone may modify the rhythm of gonadotropin-releasing-hormone (GnRH) secretion at the hypothalamic level, resulting in LH pulses of high amplitude and low frequency that maintain the activity of the corpus luteum.

After 8–9 d, in the absence of a pregnancy, involution changes start; luteinized granulosa cells decrease in size, accumulate lipids, and show degenerative nuclear changes. There is pro-

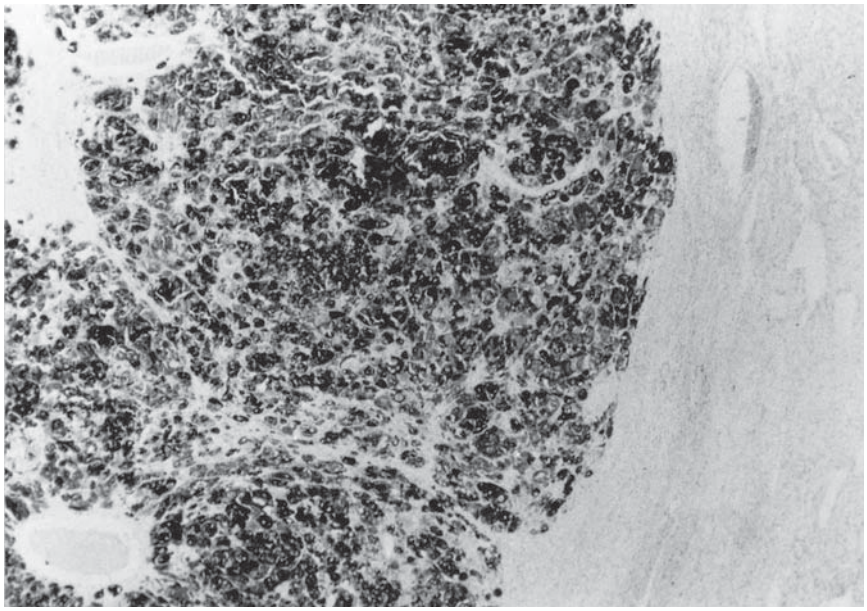

Figure 3 Strong staining for α-inhibin in a hilus cell tumor.

gressive fibrosis and transformation into a hyalinized corpus albicans. If the cycle has resulted in a pregnancy, embryonic gonadotropin (human chorionic gonadotropin [hCG]) provides the stimulus necessary to maintain the corpus luteum until the trophoblast is able to secrete enough progesterone to support pregnancy.

OTHER STRUCTURES It is important to note there are additional cell types (other than the epithelial surface, the ovarian stroma, and the sex cord elements) that may be responsible for different endocrine lesions of the ovary.

Hilus cells are typically found in the medial and lateral poles of the hilus and in the mesoovarium, often associated with nerves. They are very similar to testicular Leydig cells, and contain abundant eosinophilic cytoplasm, lipochrome pigment, and occasional Reinke crystalloids. Hilus cells may undergo hyperplasia during peri- and postmenopause.

Ectopic adrenal rests may be found in the mesoovarium. They are composed of large, vacuolated cells that resemble cortical adrenal cells. They are subjected to control from the pituitary gland through adrenocorticotropic hormone (ACTH) stimulation.

Rete ovarii is similar to the rete testis and consists of tubules with intraluminal projections.

IMMUNOHISTOCHEMICAL MARKERS The ovarian surface epithelium shares immunohistochemical markers with the mesothelial cells and the cells derived from the müllerian duct. It shows immunoreactivity for cytokeratins of low molecular weight, CA 12.5, and calretinin.

The vast majority of the other ovarian cell elements share some immunohistochemical markers, as all of them have the ability to produce steroid hormones. In general, any steroid-hormone-producing cell may exhibit positivity for α-inhibin, müllerian-inhibiting substance, Melan-A (MART-1), and calretinin. Some cell types are also positive for CD99. These markers are very important in the distinction between sex cord stromal and steroid cell tumors from epithelial–stromal or metastatic neoplasms. By far, α-inhibin is the most useful of them [3] (Fig. 3).

Inhibin and inhibin-related peptides (activin and follistatin) are gonadal hormones involved in gonadotropic regulation and the control of ovarian folliculogenesis [4]. The most important function of inhibin is the suppression of the synthesis and secretion of pituitary FSH. However, it also plays an important role as local modulator of folliculogenesis; it positively regulates androgen production by theca cells. Activin is a functional antagonist to inhibin in many cellular tissues, and follistatin is an inhibin/activin-binding protein that bioneutralizes the function of activin in many tissues. Inhibin-A and -B are heterodimers consisting of an α-subunit and a β-A or β-B subunit linked by disulfide bonds. In contrast, activins are homodimers of two β-subunits. The α-subunit of inhibin is expressed in granulosa and theca cells of normal ovaries as well as in immature Sertoli and Leydig testicular cells. Extragonadal production of α-inhibin does occur but it appears limited to a few tissues, such as the adrenal gland, placenta, pituitary, and central nervous system.

In the ovary, α-inhibin immunoreactivity has been found to be very useful, particularly in the distinction between sex cord stromal tumors and carcinomas [5–8]. The possible use of α-inhibin as a tumor marker was suggested after the observation that serum immunoreactive inhibin levels were markedly raised in the sera of patients with granulosa cell tumors and Sertoli–Leydig cell tumors of the ovary [9].

α-Inhibin usually is not expressed in the epithelial neoplastic component of ovarian carcinomas. Negative results were obtained in a total of 204 tumors studied in seven different series [3], although positive immunostaining was detected in occasional epithelial tumors. Interestingly, the luteinized nonneoplastic stromal cells that frequently surround clusters of

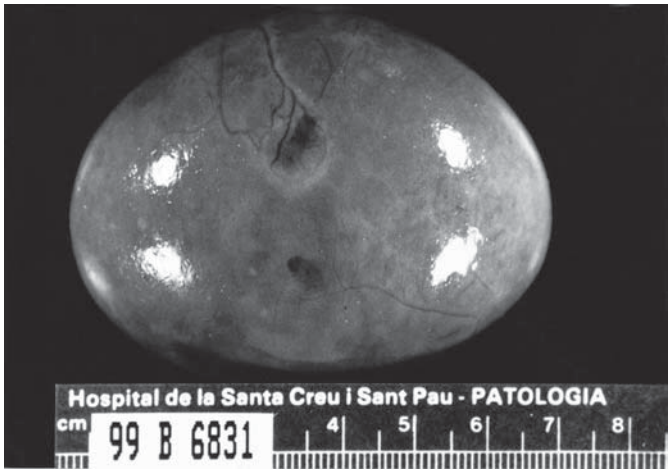

Figure 4 Gross appearance of a follicle cyst in a neonate.

Figure 5 Gross appearance of a corpus luteum cyst that ruptured and caused an intraabdominal hemorrhage.

neoplastic cells in so-called ovarian tumors with functioning stroma are frequently immunoreactive for α-inhibin [10].

TUMORLIKE LESIONS AND FUNCTIONAL CYSTS

FOLLICLE CYST Follicle cysts contain serous or hemorrhagic fluid, and are lined by granulosa cells, theca cells, or both. They usually occur in women in the reproductive age group, particularly around menarche and menopause [11]. However, they can also occur in neonates, as a result of stimulation from maternal hCG (Fig. 4). Follicle cysts are usually asymptomatic, but occasionally may be associated with hyperestrogenism, menstrual irregularities, or hemoperitoneum secondary to rupture. In neonates and children, they can produce isosexual pseudoprecocity (precocity without ovulation). Follicle cysts may coexist with polyostotic fibrous bone dysplasia, in the setting of the McCune–Albright syndrome, which is now recognized to be secondary to mutations located in exons of *GNAS1* that encode the α-subunit of the stimulatory G protein ($G_{s\alpha}$) [12].

CORPUS LUTEUM CYST Corpus luteum cyst is a cystic corpus luteum with a diameter of >3 cm. It may be associated with menstrual irregularities and, like follicle cysts, may rupture and cause intraabdominal hemorrhage (Fig. 5).

OVARIAN REMNANT SYNDROME The ovarian remnant syndrome is a clinicopathologic entity that usually occurs in women previously operated for ovarian endometriosis with subsequent peritoneal adhesions. The patients have a presumably total bilateral oophorectomy, but they present with abdominal pain and vaginal bleeding several weeks or months after surgery. A laparoscopy or a new laparotomy demonstrates the presence of residual ovarian tissue surrounded by hemorrhage and fibrosis [11].

POLYCYSTIC OVARIAN SYNDROME (PCOS) PCOS is a clinicopathologic disorder of unknown, probably heterogeneous etiology, characterized by chronic anovulation, hyperandrogenism, and enlarged polycystic ovaries. PCOS affects between 5% and 10% of women of reproductive age [13].

A consensus definition of PCOS was reached in 1990, under NIH auspices. According to this consensus effort, PCOS is defined by the presence of hyperandrogenism and ovulatory dysfunction, after exclusion of other specific disorders [14,15]. This definition is exclusively clinical; that means that the presence of enlarged ovaries with many subcapsular cysts, demonstrated by echography or gross inspection, is not a requirement for the diagnosis. In other words, there is not a complete concordance between the current definition of PCOS and what echographists or pathologists understand as PCOS. In some cases, patients fulfill the clinical criteria and present with enlarged ovaries with multiple cysts, but in other patients, the presence of enlarged ovaries with multiple cysts is not associated with the clinical manifestations of the disease. Determining the etiology of PCOS has proven elusive, but recent evidence supports a role for insulin resistance in the pathogenesis of this disorder.

The endocrine framework of PCOS is heterogeneous and corresponds to the multifactorial origin of the syndrome. As mentioned previously, the ultrasonographic and pathological manifestations of PCOS reflect a perversion of the normal process of follicular development. Variations in the stimulation of theca cells may cause a subsequent increase of androgen secretion that modifies the follicular microenvironment and disturbs follicular growth. Two main different pathways are regarded as potential promoters of this abnormal thecal activity: (1) disturbed response to LH stimulation and (2) hyperinsulinemia. For example, reduction in circulating LH, by administration of oral contraceptives or GnRH agonists, induces a clear decrease in androgen serum levels. Similarly, a fall in androgen serum levels with restoration of ovulatory cycles has resulted from improvement of insulin sensitivity in some patients. Moreover, a familial association with autosomal recessive and dominant patterns of inheritance has been reported [16]. Several different genes have been proposed as candidates; some of them related to either abnormal steroidogenesis or insulin signaling [17].

The stimulatory action of LH can be enhanced or inhibited by autocrine or paracrine mechanisms, by several modulators. However, a desensitization system is conducted by LH itself in a dose- and time-related manner. This down-regulation occurs

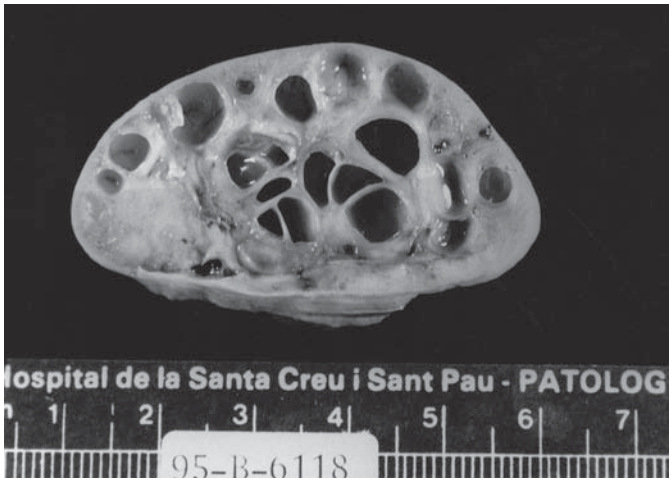

Figure 6 Gross appearance of a gonad in a patient with polycystic ovarian syndrome.

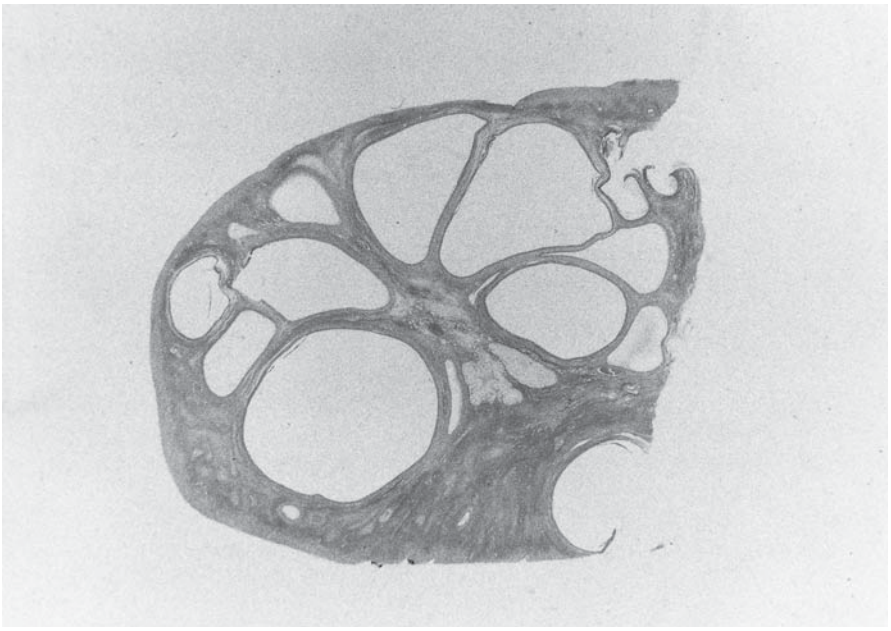

Figure 7 Low-power view of a polycystic ovary.

at the receptor and postreceptor levels by several different mechanisms (lower receptor number and sensibility, inhibition of the steroidogenetic activity); all of them may be dysregulated in patients with PCOS.

Hyperinsulinemia has also been suggested to play a major role in PCOS. It is detected in 20–60% of PCOD patients. A correlation between increased basal and stimulated insulin and androgens has been demonstrated [18,19]. In PCOS patients, hyperinsulinemia has been related to insulin resistance, and to specific insulin postreceptor defects in the phosphorylation of tyrosine and serine residues [20,21].

From a pathologic viewpoint, PCOS is characterized by a bilateral enlargement of the ovaries with multiple subcapsular follicle cysts (Figs. 6 and 7) with a prominent theca layer (follicular hyperthecosis). The subcapsular connective tissue is typically hyalinized, a feature that has been attributed to the high serum levels of androgens (Fig. 8).

Although hyperandrogenism is the most characteristic endocrine manifestation of PCOS, several patients present with hyperestrogenism as a result of transformation of androgens into estrogens by aromatization in peripheral fat. In these cases, hyperestrogenism may result in the development of hyperplasia or carcinoma of the endometrium, particularly in premenopausal patients.

STROMAL HYPERPLASIA AND STROMAL HYPERTHECOSIS Stromal hyperplasia is a condition characterized by a bilateral enlargement of the ovaries secondary to an increase in the amount of ovarian cortical stroma. The term stromal hyperthecosis is used for cases in which the stromal hyperplastic tissue contains numerous luteinized stromal cells (Fig. 9).

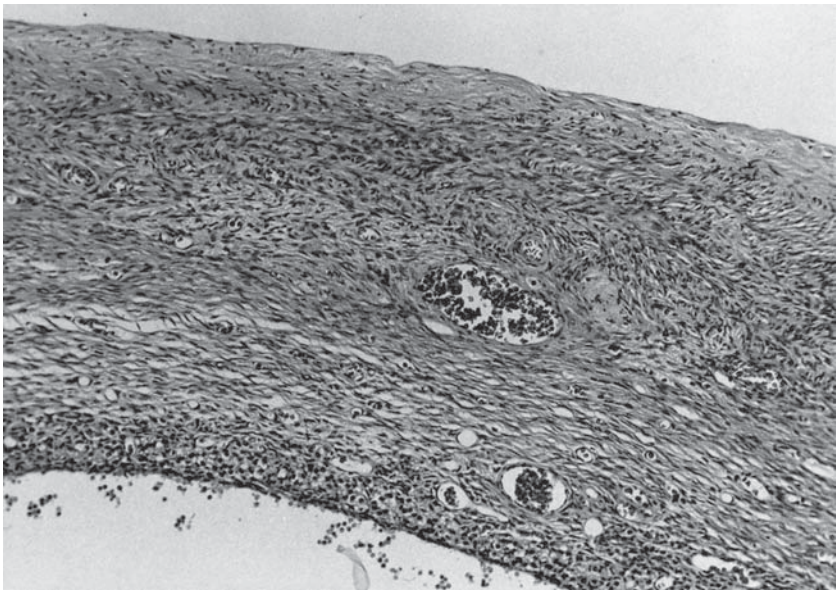

Figure 8 Microscopic appearance of a polycystic ovary. A follicle cyst is lined by a thin granulosa cell layer, and a prominent theca cell layer. Notice the presence of hyalinization in the subcapsular connective tissue.

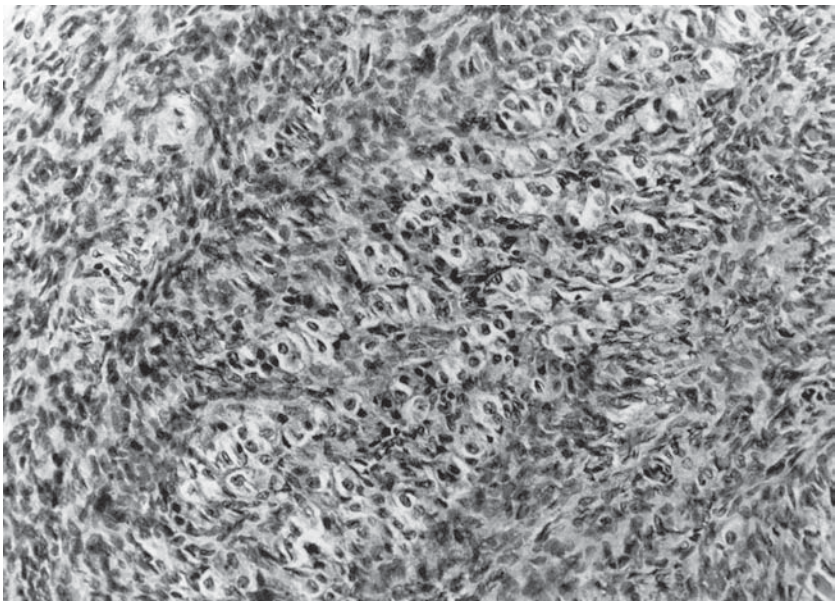

Figure 9 Numerous luteinized stromal cells in ovarian hyperthecosis.

Both stromal hyperplasia and stromal hyperthecosis may occur in pre- and postmenopausal women. In young women, stromal hyperthecosis exhibits many similarities with PCOS. In fact, some patients may show overlapping features between both entities. In these patients, stromal hyperthecosis usually manifests with hyperandrogenism, but hyperestrogenism with development of endometrial hyperplasia or carcinoma may occur (Fig. 10). Like PCOS, stromal hyperthecosis is somehow related to insulin resistance, and for that reason, it is usually associated with acanthosis nigricans in the skin (the so-called HAIR-AN syndrome) [22].

In postmenopausal woman, stromal hyperplasia and hyperthecosis may be associated with steroid hormone production. In some cases, the hirsutism and virilization may be even more severe than those associated with sex cord stromal or steroid cell tumors. On the other hand, it has been suggested that stromal hyperplasia and hyperthecosis may be the initial source for steroid hormone production in postmenopausal women with hyperestrogenism. It has been shown that the ovarian stroma of patients with endometrial carcinoma frequently contain clusters of luteinized stromal cells.

MASSIVE EDEMA AND FIBROMATOSIS Massive edema is characterized by enlargement of one or two ovaries, because of the accumulation of edema fluid. Massive edema is usually secondary to torsion (Figs. 11 and 12). In some cases, stromal lutein cells may become activated, and the patients may present

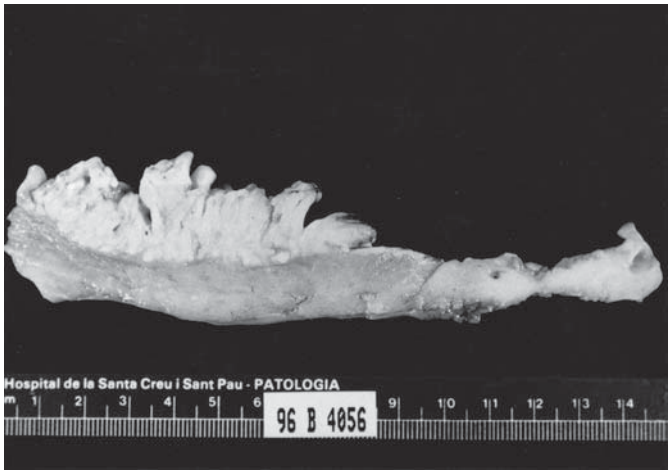

Figure 10 An endometrioid carcinoma of the endometrium in a premenopausal woman with bilateral ovarian hyperthecosis.

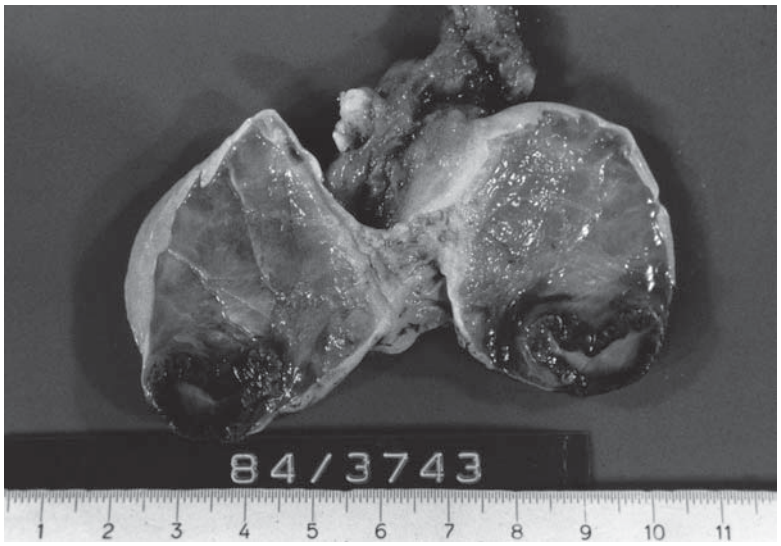

Figure 11 Gross appearance of massive edema. The ovary is enlarged and contains abundant watery fluid.

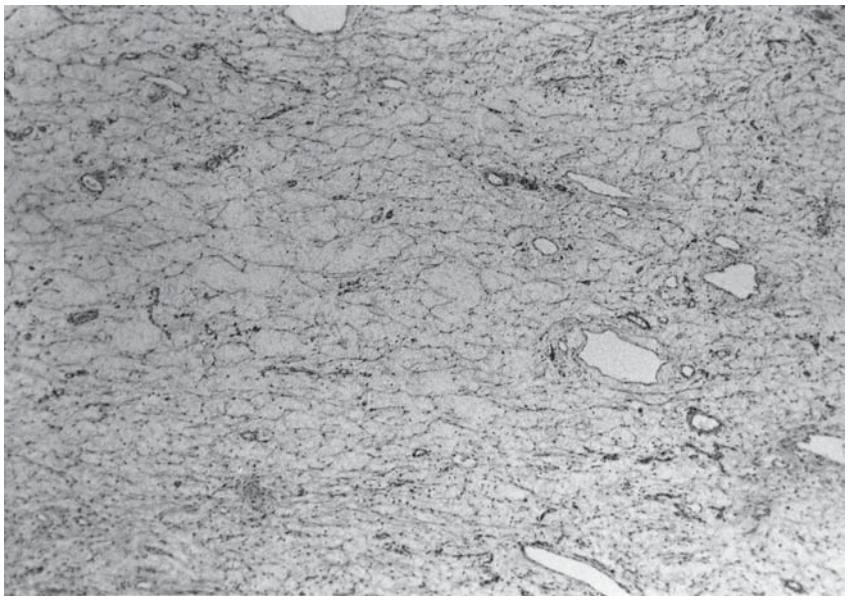

Figure 12 Microscopical appearance of ovarian massive edema.

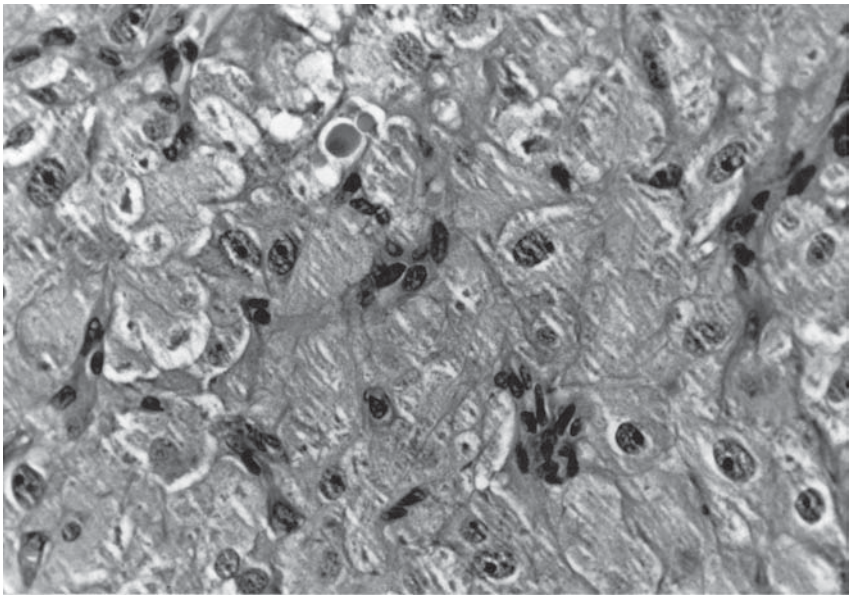

Figure 13 Eosinophilic hyaline bodies in a corpus luteum of the pregnancy.

with symptoms secondary to hyperestrogenism or virilization. Several investigators have suggested that massive edema and ovarian fibromatosis are two ends in the spectrum of the same disorder [23].

OVARIAN LESIONS ASSOCIATED WITH PREGNANCY

CORPUS LUTEUM OF THE PREGNANCY The corpus luteum of the pregnancy is very similar to that of the menstruation. However, it shows two distinctive histological features: eosinophilic hyaline bodies (Fig. 13) and calcifications [24].

PREGNANCY LUTEOMA Pregnancy luteoma is a tumorlike disorder that occurs during pregnancy as single or multiple nodules, typically in the second half of pregnancy. It is frequently associated with multiparity. The nodules contain abundant lutein cells. Pregnancy luteomas are usually asymptomatic, incidental finding at the time of cesarean section or tubal ligation. However, they may be associated with hirsutism or virilization in the mother or female infants. Pregnancy luteoma is a benign tumorlike lesion that regresses spontaneously after termination of pregnancy [24].

HYPERREACTIO LUTEINALIS Hyperreactio luteinalis is also a tumorlike lesion that occurs in pregnant patients with high hCG levels (e.g., gestational throphoblastic disorders). It can also occur in patients after administration of drugs for induction of ovulation. Hyperreactio luteinalis is characterized by a bilateral ovarian enlargement and numerous thin-walled, luteinized follicle cysts. It may coexist with pregnancy luteomas [24].

LARGE SOLITARY LUTEINIZED FOLLICULAR CYST OF PREGNANCY AND PUERPERIUM This is a unilateral and unilocular cyst that contains watery fluid, frequently detected at the time of cesarean section or on routine physical examination during the puerperium. It is characteristically lined by large, eosinophilic, luteinized cells with bizarre nuclei. It typically occurs during pregnancy or puerperium (Figs. 14 and 15). It can reach a large size and pose problems with malignancy. The major important differential diagnosis is with a unilocular cystic granulosa cell tumor [25].

GRANULOSA CELL PROLIFERATION OF PREGNANCY These are characterized by clusters of granulosa cells, frequently associated with atretic follicles. They are incidental findings, unassociated with endocrine manifestations, but they may pose problems in differential diagnosis [24,26].

ENDOCRINE SYNDROMES ASSOCIATED WITH OVARIAN TUMORS

Ovarian neoplasms may be accompanied by a great variety of endocrine manifestations. By far, hyperandrogenism and hyperestrogenism are the most frequent of these [27].

The normal ovary contains a large variety of cells that are able to secrete steroid hormones, but there is a great degree of overlapping in the production of estrogens and androgens. In fact, some cell types produce androgens, other cells secrete estrogens, and others both substances. For instance, granulosa cells usually secrete estrogens, while theca cells may produce either androgens or estrogens. The spindle cells of the ovarian stroma may also secrete either estrogens or androgens. On the other hand, hilus cells predominantly produce androgens. Moreover, estrogens can be produced as a result of aromatization of androgens.

The hormonal profile of ovarian tumors reflects the overlapping in the production of steroid hormones in the normal cell counterparts. Some tumors are associated with hyperestrogenism, other tumors show hyperandrogenism, but the vast majority may be associated with both types of hormone excess.

HYPERESTROGENISM The ovarian tumors most frequently associated with hyperestrogenism are thecoma, granulosa cell

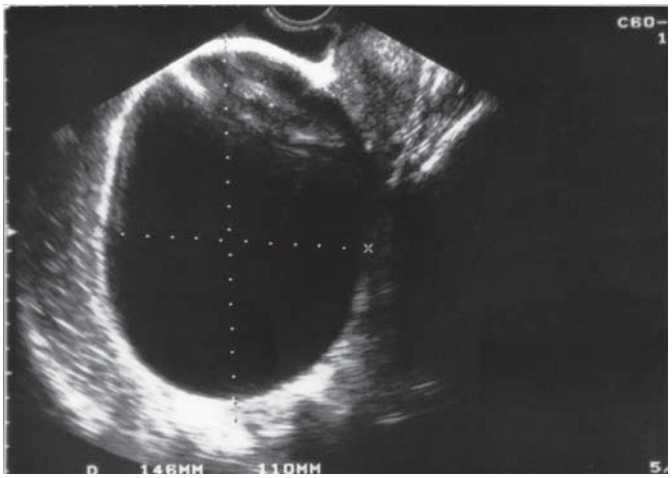

Figure 14 Ultrasonographic scan of a large solitary luteinized follicular cyst of pregnancy and puerperium.

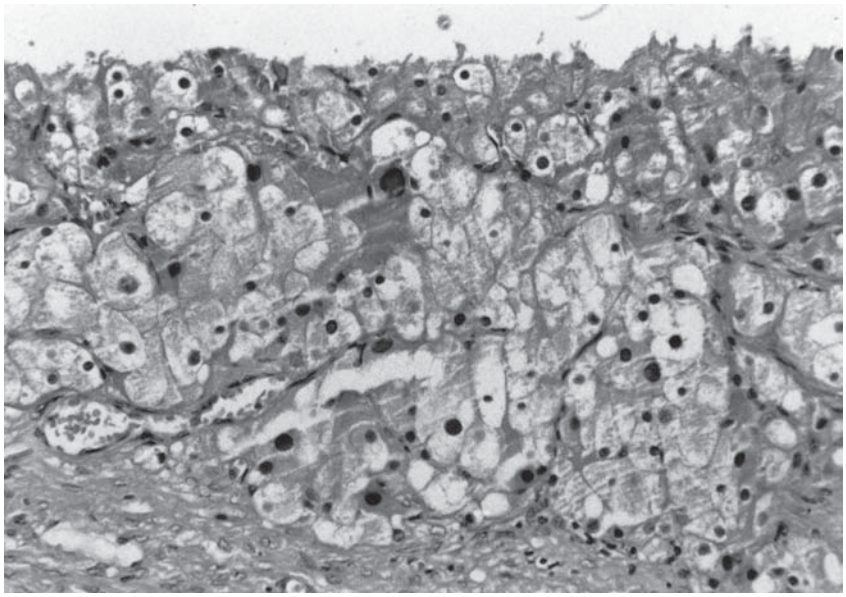

Figure 15 Microscopic appearance of a large solitary luteinized follicular cyst of pregnancy and puerperium; prominent luteinization and bizarre nuclei.

tumor, stromal luteoma, and the group of ovarian tumors with functioning stroma. The manifestations of hyperestrogenism are frequently subtle. Children may exhibit isosexual pseudoprecocity, and premenopausal women can present menstrual irregularities or amenorrhea. Postmenopausal women typically have vaginal bleeding; in these patients, an endometrial biopsy may demonstrate the presence of endometrial hyperplasia, or cervicovaginal smears can show an abnormal estrogenic pattern.

HYPERANDROGENISM Hyperandrogenism can be found in association with several types of ovarian tumors. Pure Sertoli cell tumors, Sertoli–Leydig cell tumors, and hilus cell tumors are frequently associated with high androgen serum levels. However, steroid cell tumors not otherwise specified (NOS), thecomas, granulosa cell tumors (particularly the cystic subtype), stromal luteomas, and the ovarian tumors with functioning stroma may also present with high levels of androgens. The clinical appearance of hyperandrogenism is quite typical. Patients may show various degrees of masculinization (acne, hirsutism, temporal balding, deepening of the voice, and enlargement of the clitoris). Serum levels of testosterone and androstenedione may be elevated. In contrast to virilizing adrenal tumors, the urinary 17-ketosterone levels are usually (but not always) normal, and the serum levels of dehydroepiandrostenedione sulfate are usually normal.

It is worth emphasizing that, particularly in postmenopausal women, hyperandrogenism with hirsutism and virilization may be due to non-neoplastic disorders such as stromal hyperthecosis or massive edema.

OVARIAN TUMORS WITH FUNCTIONING STROMA The ovarian tumors with functioning stroma (OTFS) are an interesting group of tumors that can present with hyperestrogenism or hyperandrogenism. OTFS are defined as the ovarian tumors, other than sex cord stromal or steroid cell tumors, whose stroma is consistent with steroid hormone secretion, and are

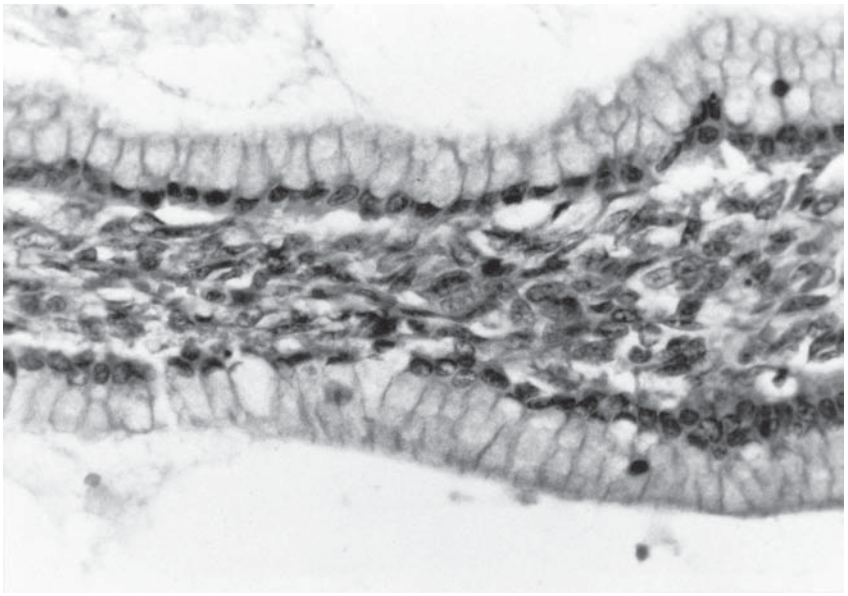

Figure 16 Stromal luteinization in a mucinous cystadenoma with functioning stroma.

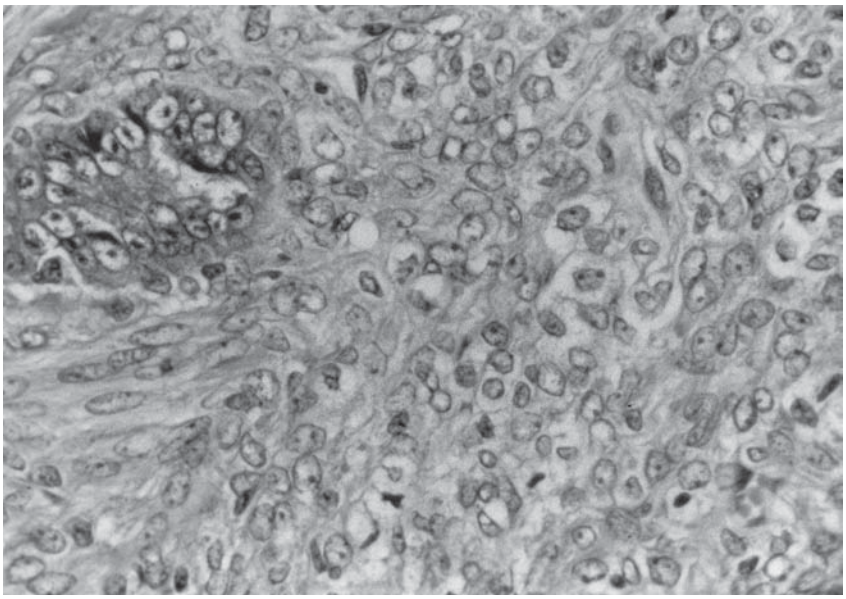

Figure 17 Luteinized stromal cells in an endometrioid carcinoma with functioning stroma.

associated with estrogenic, androgenic, or progestagenic manifestations. In this group of tumors, steroid hormones are not produced by tumor cells; they are secreted by ovarian stromal cells under the stimulus of tumor cells.

From a pathogenetic viewpoint, OTFS may fall in three groups: (1) tumors that contain syncytiotrophoblastic cells that produce hCG and stimulate the ovarian stroma (e.g., ovarian dysgerminoma with syncytiotrophoblastic cells); (2) tumors occurring during pregnancy; and (3) tumors that do not contain syncytiotrophoblastic cells and do not occur during pregnancy. By far, the third group is the most common.

Although any type of primary or secondary ovarian tumor may show activation of the stroma, the vast majority of OTFS of the third group are benign, borderline, or malignant mucinous tumors and ovarian metastases from primary gastrointestinal carcinomas. Interestingly, struma ovarii may also show activation of the ovarian stroma, particularly at the periphery of the tumor [28]. In OTFS, the stroma has a very distinctive appearance; it can either be condensed (Fig. 16) or contain clusters of luteinized stromal cells (Fig. 17). The vast majority of OTFS are not associated with overt clinical endocrine manifestations. However, the patients frequently show subclinical signs of hyperestrogenism, such as elevated serum or urinary levels of steroid hormones, and/or subtle changes in target tissues (endometrium, cervix, breasts) [29]. Nevertheless, it is worth mentioning that some patients may present with marked

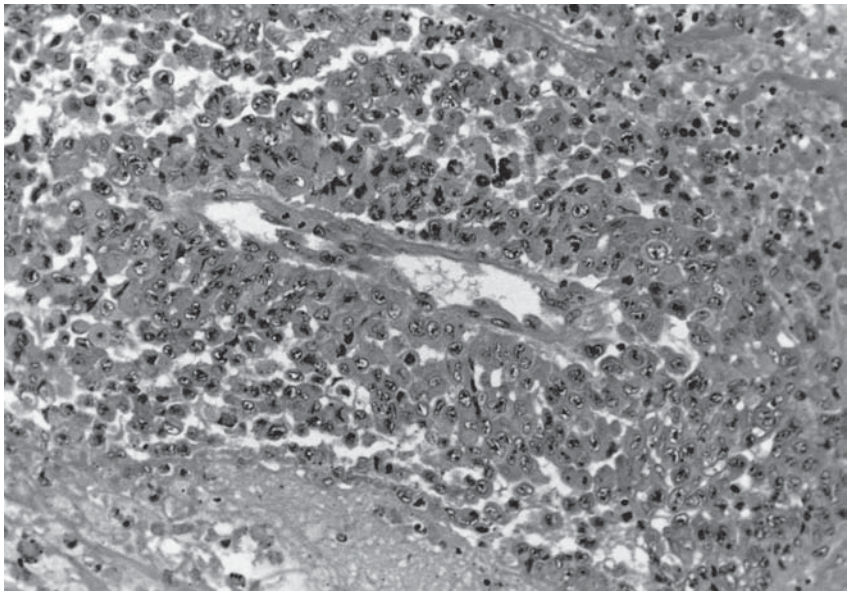

Figure 18 Microscopic appearance of a small cell carcinoma associated with hypercalcemia.

clinical evidence of hormone excess. For example, sometimes, patients with Krukenberg tumors present with virilization as an initial symptom, as a result of activation of the ovarian stroma, leading to a preoperative misinterpretation as sex cord stromal ovarian tumor [27].

The reason for the activation of the stroma in the third group of OTFS is unclear. Several investigators suggested that the ectopic production of hCG by tumor cells could be the explanation of the stromal activation in these cases. In 1990, a study found a correlation between the production of hCG by tumor cells, determined by immunohistochemistry with several poly- and monoclonal antibodies and the presence of condensation or luteinization of the ovarian stroma in a series of 100 consecutive ovarian tumors [30]. However, it is feasible that other factors may contribute to this phenomenon.

HYPERCALCEMIA Hypercalcemia is a frequent paraneoplastic syndrome in tumors from different organs. It may also be found among ovarian tumors. The two types of ovarian tumors that are most frequently associated with elevated serum levels of calcium are the small-cell carcinoma (the so-called hypercalcemic small-cell carcinoma) and clear-cell carcinoma. Rare examples of ovarian dysgerminoma, serous tumors, or squamous cell carcinomas arising from preexisting dermoid cysts have also been reported in association with hypercalcemia.

The small-cell carcinoma of the ovary is a distinctive malignant tumor type that usually occurs in children and young women and is associated with elevated serum levels of calcium in approximately two thirds of the patients. It is characterized by poorly differentiated small cells with densely hyperchromatic nuclei and scanty cytoplasm (Fig. 18), although larger cells with abundant cytoplasm may be observed in approximately half of the cases. Distinctive folliclelike structures containing eosinophilic fluid may be present, which may pose problems in differential diagnosis with juvenile granulosa cell tumors. The lack of the typical follicles of granulosa cell tumor, absence of a thecal component, higher mitotic rate and negativity for α-inhibin are useful tools in differential diagnosis.

Attempts to demonstrate parathyroid hormone (PTH) immunostaining in these tumors were unsuccessful in the vast majority of cases [31]. In 1994, a study demonstrated positive immunostaining for PTH-related peptide in five of seven cases of small cell carcinoma, clearly indicating that PTH-related peptide is the most possible candidate to be responsible for the hypercalcemia that is frequently associated with ovarian small cell carcinomas [32].

OTHER ENDOCRINE SYNDROMES Ovarian tumors may also be associated with several other endocrine syndromes such as hyperthyroidism, carcinoid syndrome, hypergastrinemia, Cushing's syndrome, hypoglycemia, hyperaldosteronism, or hyperprolactinemia [27].

SEX CORD STROMAL TUMORS

Sex cord stromal tumors are infrequent, and represent 8% of all primary ovarian tumors. Sex cord stromal tumors contain granulosa cells, theca cells, Sertoli cells, Leydig cells, and spindle-shaped cells of gonadal stromal origin, either separately or admixed.

ADULT GRANULOSA CELL TUMOR Adult granulosa cell tumors (AGCTs) represent 1–2% of all ovarian tumors. They usually occur in peri- and postmenopausal women, and are frequently accompanied by estrogenic manifestations. In some cases, estrogenic symptoms may be subclinical, and they present as a palpable mass, or as hemoperitoneum secondary to rupture. AGCTs are usually unilateral, and solid and cystic. The solid component frequently shows a yellow discoloration [33,34]. Occasional tumors are cystic, and they are frequently associated with virilization [35].

AGCTs are composed of granulosa cells, although they frequently contain theca or spindle stromal cells. Tumor cells are typically irregular and contain grooved nuclei (Fig. 19), haphaz-

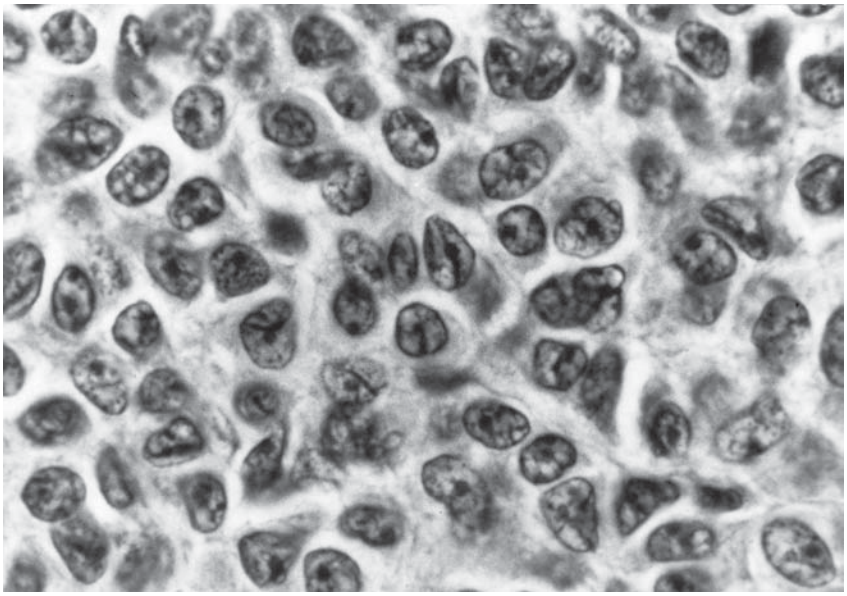

Figure 19 Nuclear grooves in a typical adult granulosa cell tumor.

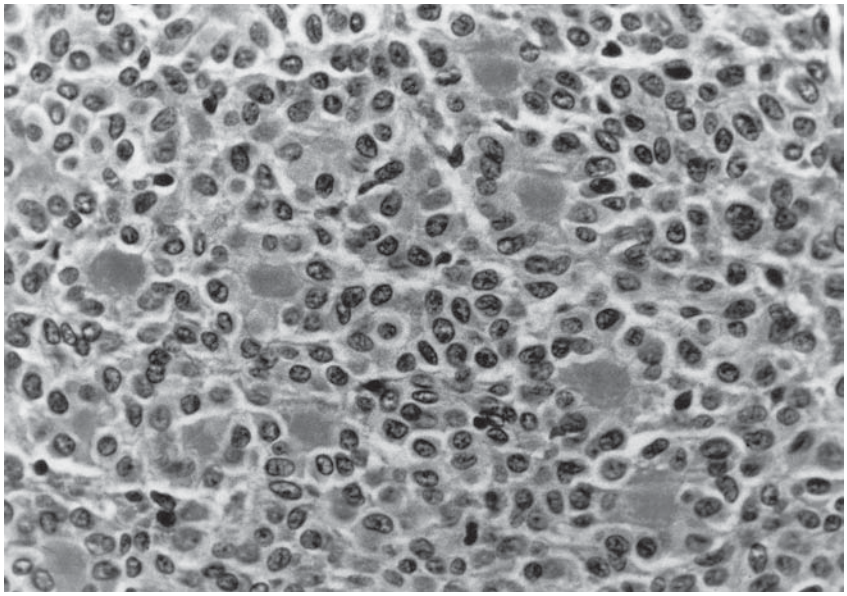

Figure 20 Call–Exner bodies in an adult granulosa cell tumor.

ardly oriented to one another. Scattered cells with pleomorphic, large, atypical nuclei can be detected in approx 2% of the tumors. AGCTs may show a wide variety of architectural patterns that may be found separately or in combination. They include the microfollicular (the most characteristic), macrofollicular, insular, trabecular, solid-tubular, gyriform, and diffuse (sarcomatoid). Some tumors show rosettelike structures that resemble the Call–Exner bodies of the graafian follicles (Fig. 20).

AGCTs are potentially malignant tumors that may spread within the pelvis and lower abdomen. Recurrences usually appear within 5 yr after the initial surgery. However, late recurrences and distant metastasis are sometimes detected. The 10-yr survival rate ranges from 60% to 90%. The most important prognostic factor is the presence of metastasis or invasion of structures outside the ovary at the time of diagnosis. Less important adverse prognostic factors are tumor size (6 cm), and the presence of a predominant diffuse or sarcomatoid component [36–38]. The most important differential diagnosis is with poorly differentiated endometrioid carcinoma (Fig. 21); the presence of typical low-grade endometrioid carcinoma or adenofibromatous components, lack of nuclear grooves, and negativity for α-inhibin are good tools for the correct diagnosis. Reticulin stains are also useful [8,39].

JUVENILE GRANULOSA CELL TUMOR Juvenile granulosa cell tumors (JGCTs) are very rare and represent 5% of all granulosa cell tumors [39]. JGCT usually occurs in women <30

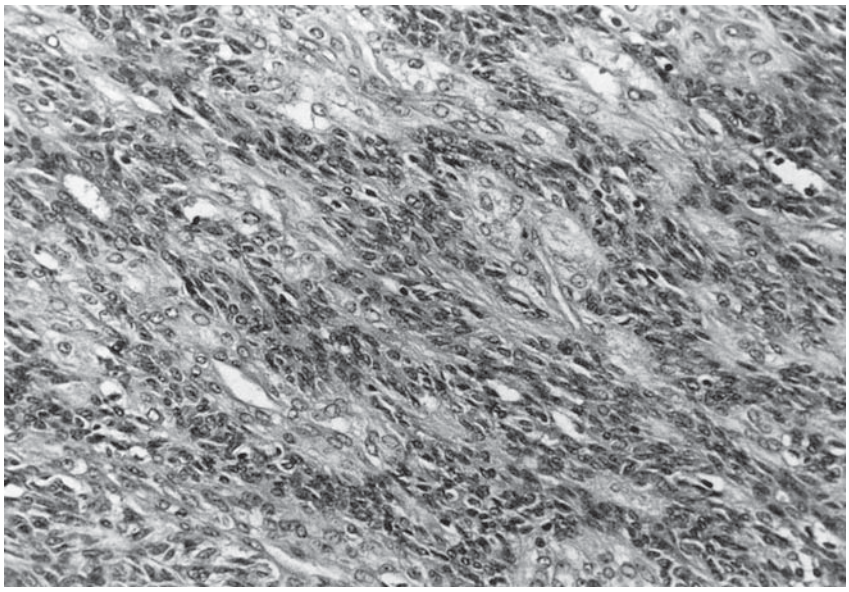

Figure 21 Microscopical appearance of an ovarian endometrioid carcinoma that resembled a sex cord stromal tumor. Tumor cells were negative for α-inhibin. Typical focus of low-grade endometrioid carcinoma were found after an intense sampling.

Figure 22 Gross appearance of an ovarian fibroma.

yr of age, but it may occur in older patients. When JGCT occurs before puberty, it may be accompanied by isosexual pseudoprecocity, because of estrogen secretion by tumor cells.

JGCTs are usually unilateral, cystic, and solid. The microscopic picture is different from AGCT. Tumor cells are less mature, lack the typical nuclear grooves, and may have a luteinized appearance. Tumor cells are frequently arranged in diffuse sheets or nodules, and show poorly defined follicular spaces that contain eosinophilic or basophilic mucinous material. A theca cell component may be seen. Nuclear atypia and high mitotic index are two misleading features.

Stage is also a good prognostic indicator. Stage I tumors are usually associated with good prognosis. The most important differential diagnosis is the small cell carcinoma of the hypercalcemic type, which also occurs in children and young patients, and may show pseudofollicular spaces. However, the appearance of the cells and positivity for α-inhibin are good tools for diagnosis.

THECOMA AND FIBROMA The tumors of the thecoma–fibroma group account for almost 90% of sex cord stromal tumors. They are composed of cells resembling theca interna (thecoma) or spindle cells of stromal origin (fibromas) (Fig. 22). Both cell elements may coexist in variable proportion in some tumors [11].

Typical thecomas usually occur in postmenopausal women, and are associated with estrogenic manifestations. Luteinized thecomas (thecomas with prominent luteinization) occur in younger patients and may be accompanied by androgenic symptoms.

Thecomas and fibromas are unilateral, solid tumors, with a white to yellow discoloration. The only bilateral luteinized thecomas are those associated with sclerosing peritonitis [40]. Occasional fibromas may contain foci of sex cordlike elements.

Figure 23 Gross appearance of a Sertoli–Leydig cell tumor. In this case, the patient presented with hyperestrogenism and endometrial hyperplasia.

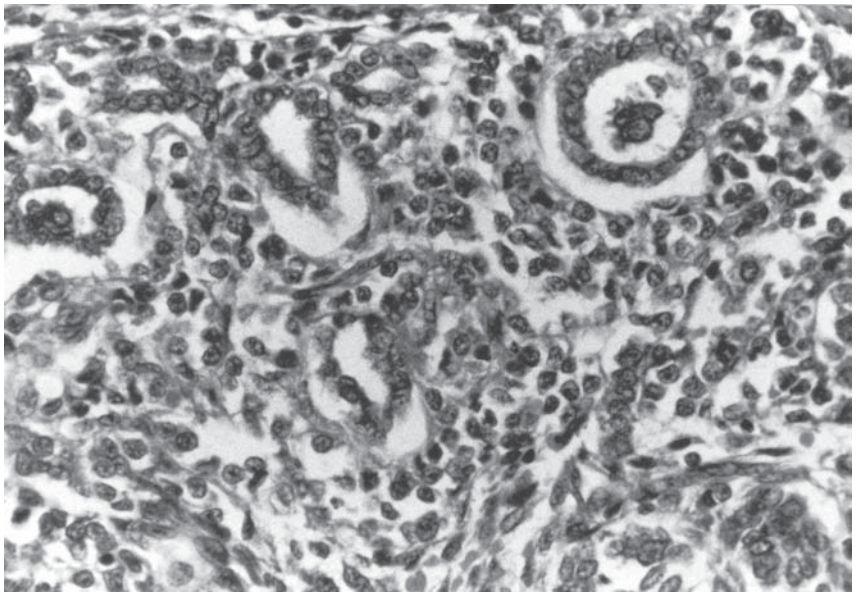

Figure 24 Microscopic appearance of a Sertoli–Leydig cell tumor of intermediate differentiation.

SERTOLI–STROMAL CELL TUMORS Sertoli–stromal cell tumors are very infrequent. In some rare cases the tumors are composed of tubules of Sertoli cells separated by stroma that do not contain Leydig cells (Sertoli cell tumors) [41].

The most typical tumors show a mixture of Sertoli cells arranged in tubules or cords, and large, eosinophilic Leydig cells, and they are designated as Sertoli–Leydig cell tumors (SLCTs). SLCTs are unilateral (Fig. 23), and usually occur in women of reproductive age. They are usually associated with virilization, but may be nonfunctioning, or even present estrogenic manifestations [42–44].

SLCTs may show a wide variety of microscopical patterns. Five histologic variants have been recognized:

1. Well-differentiated tumors, characterized by tubules of Sertoli cells surrounded by a nonspecific fibrous stroma with nests of Leydig cells with occasional Reinke crystalloids [45].
2. Tumors of intermediate differentiation that contain abortive tubules and cords of immature Sertoli cells separated by a fibrous stroma and Leydig cells (Fig. 24).
3. Poorly differentiated or sarcomatoid tumors, composed of spindle cells with vague trabecular configuration.
4. SLCTs with heterologous elements. These tumors contain foci of neoplastic cartilage, mucinous glands, neuroendocrine (carcinoid) cells, and skeletal muscle [46,47].
5. Tumors with retiform pattern, exhibiting areas that resemble the rete of the ovary or the testis [48,49].

SLCTs are usually stage Ia tumors that follow a benign behavior. Recurrence is associated with poorly differentiated tumors and presence of mesenchymal heterologous elements.

SEX CORD TUMOR WITH ANNULAR TUBULES (SCTAT) SCTATs has a peculiar histologic appearance that is intermediate between granulosa and Sertoli cell tumor. It is composed of

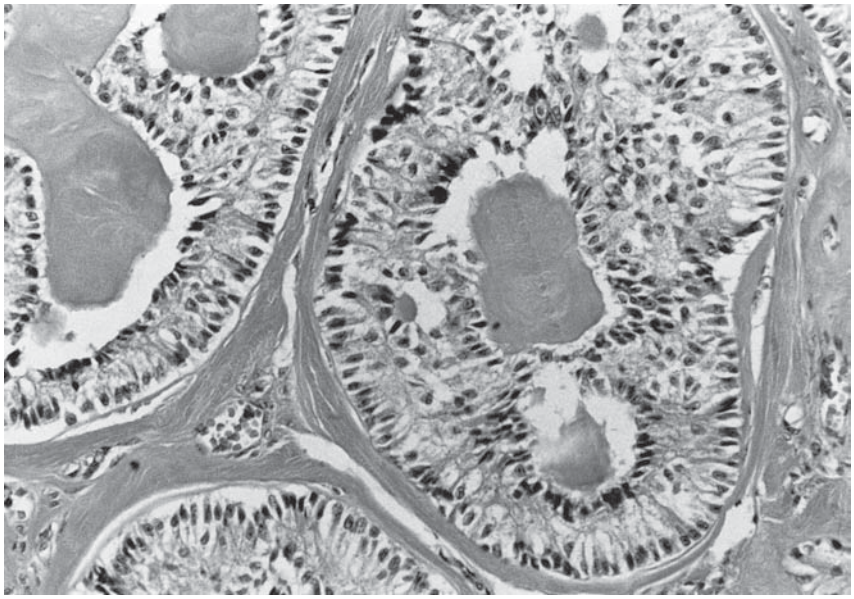

Figure 25 Microscopic appearance of a sex cord tumor with annular tubules.

complex annular tubules containing a central hyaline body of basement membrane material (Fig. 25). The vast majority of SCTATs are benign but a few are malignant [50].

One third of the SCTAT cases are associated with the Peutz–Jegher syndrome, a familial syndrome that also includes gastrointestinal hamartomatous polyps; mucocutaneous pigmentation; well differentiated adenocarcinoma of the uterine cervix; and carcinomas of the colon, pancreas, and breast [51]. The gene responsible for Peutz–Jegher syndrome was recently identified. It mapped to chromosome 19q13.3 and encoded the serine threonine kinase STK11 [52].

GYNANDROBLASTOMA This is a rare tumor that show areas of granulosa–theca cell tumor coexisting with other of Sertoli–Leydig cell tumor [11].

MOLECULAR PATHOLOGY OF SEX CORD STROMAL TUMORS Some preliminary reports suggested a role for somatic mutations in the follicle-stimulating hormone receptor in some sex cord stromal tumors, particularly granulosa cell tumors [53]. However, additional studies have not confirmed such results, which were subsequently interpreted by the authors as artifactual, due to DNA contamination [54,55].

Moreover, the fact that germline mutations in the *STK11/LKB1* gene were responsible for Peutz–Jegher syndrome [52] prompted some authors to investigate the presence of somatic mutations in this gene in sporadic sex cord stromal tumors. However, the results that were obtained do not support a role for this gene in sporadic tumors [56].

Finally, it has been shown that sex cord stromal tumors frequently exhibit a near diploid chromosome number, but also a high frequency of trisomy 12 and occasionally monosomy or trisomy X. The presence of trisomy 12 has been confirmed in different studies, and may be used in differential diagnosis (Fig. 26), by performing fluorescence *in situ* hybridization (FISH) with centromeric probes specific for chromosome 12 [57–59].

STEROID CELL TUMORS

The term steroid cell tumor encompasses a group of ovarian neoplasms that are composed of cells resembling steroid hormone secreting cells (lutein cells, Leydig cells, or adrenal cortical cells). These tumors were previously designated as lipoid cell tumors. These tumors are uncommon, and represent 0.1% of all ovarian tumors. However, they are important, because they are frequently associated with hyperestrogenism or hirsutism and virilization [11].

Steroid cell tumors include three different tumor subtypes: stromal luteoma, Leydig cell tumors, and steroid cell tumors NOS. From the immunohistochemical viewpoint, all steroid cell tumors have a common marker; all of them are positive for α-inhibin.

STROMAL LUTEOMA Stromal luteoma is a benign tumor accounting for 20% of steroid cell tumors, and usually occurs in postmenopausal women. Stromal luteoma usually is a small nodule, composed of steroid-producing cells that resemble lutein cells, that is located within the ovarian stroma. The cells are arranged in a vague organoid pattern. In some cases, the artefactual formation of irregular spaces with hemorrhage can lead to a misdiagnosis of a vascular tumor (Fig. 27). Stromal luteoma usually develops in the context of preexisting bilateral stromal hyperthecosis [60]. It is commonly associated with hyperestrogenism, although some infrequent cases have been associated with masculinization [61].

LEYDIG CELL TUMOR The Leydig cell tumor is composed of Leydig cells. The vast majority of the reported tumors are located in the hilus and arise from hyperplastic hilus (Leydig) cells; they are named hilus cell tumors. On rare occasions, Leydig cell tumors may occur within the ovarian stroma, far from the hilus; they are designated as Leydig (nonhilus) cell tumors.

Leydig cell tumors represent 20% of steroid cell tumors. They are benign, and usually occur in postmenopausal women. They are associated with various degrees of hyperandrogenism.

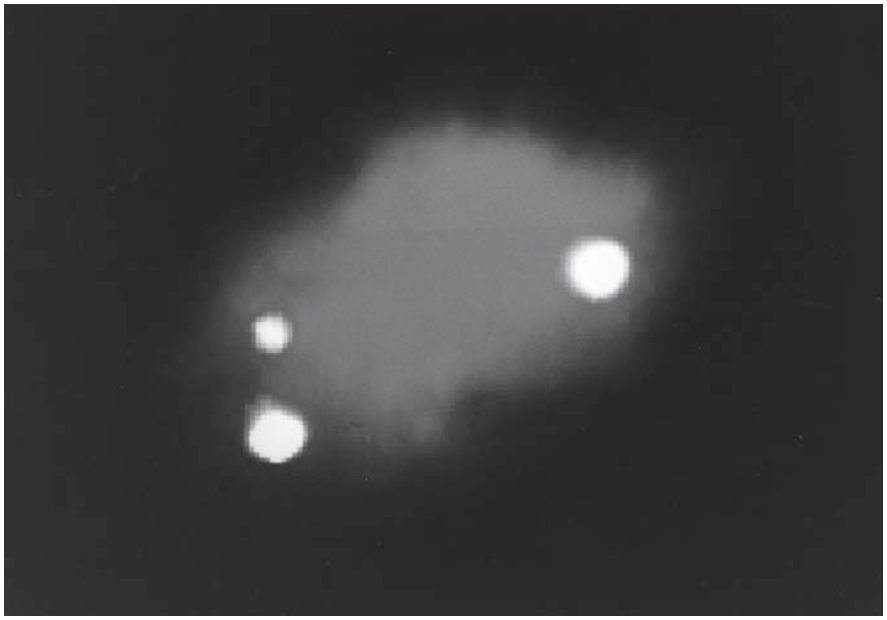

Figure 26 Trisomy 12 detected by fluoresence *in situ* hybridization (FISH) with centromeric probes in isolated cells from a Sertoli–Leydig cell tumor.

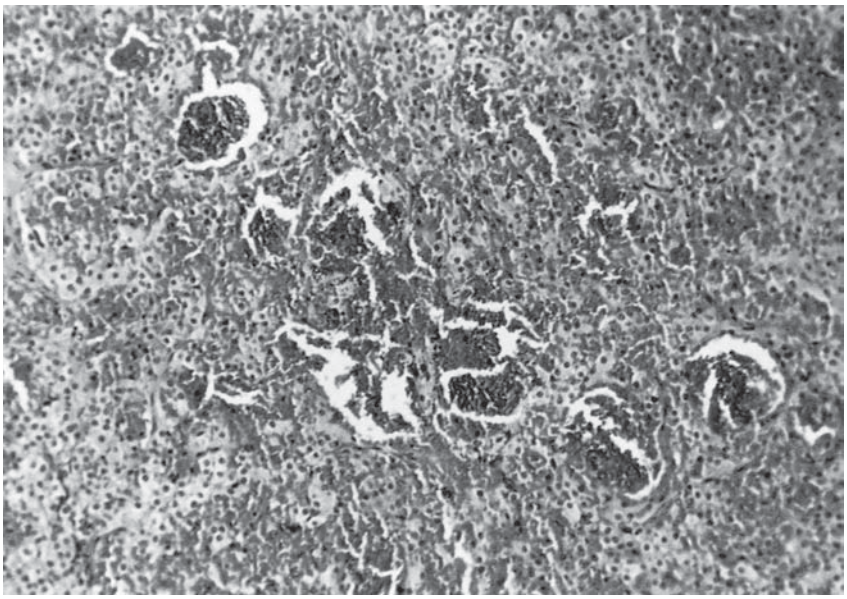

Figure 27 Irregular spaces containing blood in a stromal luteoma.

Leydig cell tumors are small nodules, usually located in the ovarian hilus, of brown coloration. From a microscopical viewpoint, they are composed of polygonal shaped cells arranged in nests, surrounded by fibrovascular septa. In some cases, Leydig cell tumors may contain cells with nuclear pseudoinclusions or marked (endocrine) nuclear pleomorphism (Fig. 28). They frequently contain Reinke crystalloids and lypochrome pigment. Leydig cell tumors with crystalloids are more frequently associated with virilization than Leydig cell tumors without crystalloids [62].

STEROID CELL TUMORS NOS Steroid cell tumors NOS is a heterogeneous group of tumors that includes the steroid cell tumors that do not fulfill the criteria for stromal luteoma or Leydig cell tumors. They represent 60% of steroid cell tumors. In one series, clinically malignant tumors accounted for 25% of the cases [63].

Steroid cell tumors NOS may show different gross and microscopical features. Some tumors are brown while others are yellow. Some tumors are composed of polygonal shaped eosinophilic cells with lypochrome, whereas others contain large cells with clear cytoplasms with abundant lipids.

Several clinicopathologic criteria need to be taken into account when considering the possibility of malignancy. Age at presentation is a good indicator or malignant behavior, as the vast majority of malignant tumors occur in women older than 51 yr. Other features to be considered are: (1) more than two mitoses per 10 HPF; (2) size >7 cm; (3) hemorrhage; and (4) the presence of marked nuclear atypia.

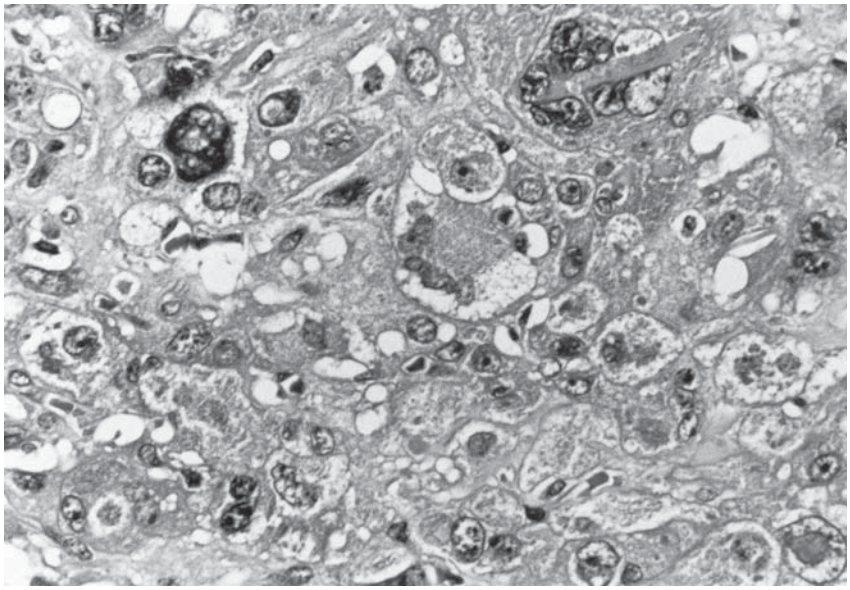

Figure 28 Marked nuclear pleomorphism and occasional Reinke crystalloids in an ovarian hilus cell tumor.

Some examples of steroid cell tumors NOS are tumors originating from ectopic adrenal cortical rests, and that may be associated with Cushing's syndrome.

MOLECULAR PATHOLOGY OF STEROID CELL TUMORS Interesting results have been obtained regarding the molecular basis of the steroid cell group of tumors. Two major molecular alterations have been proposed for these tumors.

There is some evidence suggesting a role for activating mutations of the LH receptor gene in Leydig cell tumors. In fact, activation of this receptor regulates the development and function of Leydig cells, and inactivating mutations of the LH receptor have been associated with pseudohermaphroditism and Leydig cell agenesis [64,65]. In 1999, a group of investigators identified the very same activating somatic mutation of the LH receptor gene in testicular Leydig cell tumors from three different patients [66]. The mutation consisted in the substitution of cytosine for guanine at nucleotide 1732, which encoded replacement of aspartic acid with histidine at amino acid position 578. Interestingly, a different mutation in this very same codon had been previously detected in boys with severe precocious puberty and diffuse Leydig cell hyperplasia. At least two additional studies have confirmed the presence of mutations of the LH receptor gene in Leydig cell tumors [67,68].

A different group of investigators identified activating mutations in the α subunit of G_s in three ovarian and one testicular Leydig cell tumor [69]. The heterodimeric G proteins, composed of α-, β-, and γ-subunits, are a family of proteins that link cell surface receptors for a wide variety of extracellular signals to either enzymes or ion channels, resulting in the generation of a second messenger. Various G_α proteins define different G protein trimers (G_s, G_q, G_i, G_t), each of which regulates a distinctive set of downstream signaling pathways. The activity of a trimeric G protein is regulated by the binding and hydrolysis of GTP by the G_α subunit. Mutations of the α-subunit genes that lead to constitutive activation (α_s and α_{i2}) have been associated with endocrine diseases. The α_s mutations (gsp) have been associated with pituitary adenomas, thyroid follicular adenomas, and McCune–Albright syndrome, and result in decreased intrinsic GTPase activity and accelerated cAMP production in the absence of stimulatory hormone. The activating mutations that were identified in Leydig cell tumors involved the α-subunit of the $G\alpha_{i2}$ protein, causing a sustained inhibition of adenylyl cyclase activity, and resulting in a decreased basal level of cAMP. Interestingly, mutations in this α-subunit of the $G_{\alpha i2}$ protein were also detected in sex cord stromal tumors of the ovary by one group, but the results were not confirmed by others [70].

GERM CELL TUMORS AND GONADAL DYSGENESIS

GERM CELL TUMORS Ovarian germ cell tumors represent 30% of all ovarian tumors. More than 95% of these are mature cystic teratomas (dermoid cysts), and the remaining are malignant. Malignant ovarian germ cell tumors generally occur in children and young women. Most malignant ovarian germ cell tumors occur in pure form, but a minority (10%) show a combination of different tumor types [11].

Dysgerminoma is the most common malignant germ cell tumor, but accounts for only 1% of all ovarian malignancies. It is usually a unilateral, large, solid tumor with a white to gray discoloration. It shows the typical microscopic features of testicular seminoma, with nests of large, glycogen-rich cells surrounded by fibrovascular septa with numerous lymphocytes. A granulomatous reaction is occasionally seen. Scattered syncytiotrophoblastic cells are sometimes found; they produce hCG, and are responsible for the presence of luteinized stromal cells in the adjacent ovarian stroma. As mentioned later, dysgerminoma may develop from a preexisting gonadoblastoma in the setting of pure or mixed gonadal dysgenesis.

Endodermal sinus tumor (yolk sac tumor) is almost as common as dysgerminoma in patients under the age of 20 yr. It may

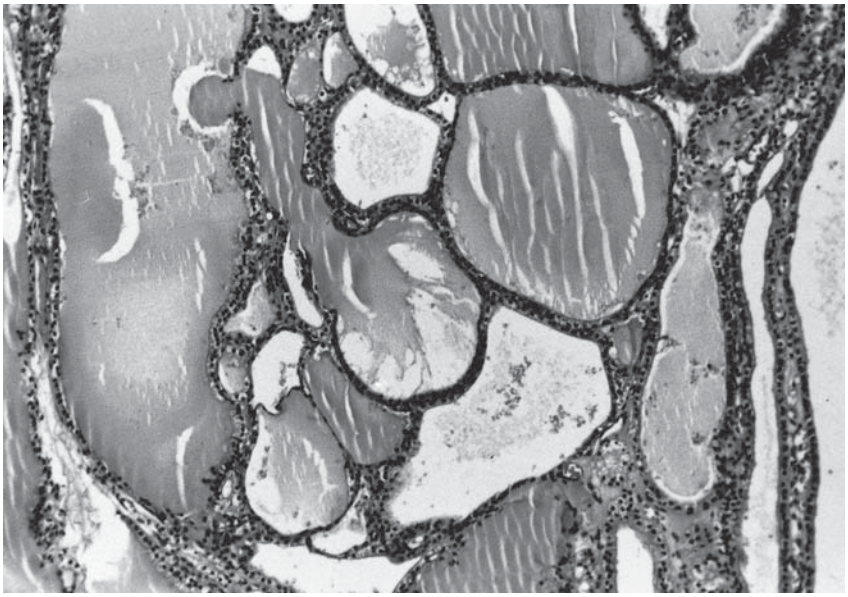

Figure 29 Thyroid follicles in a struma ovarii.

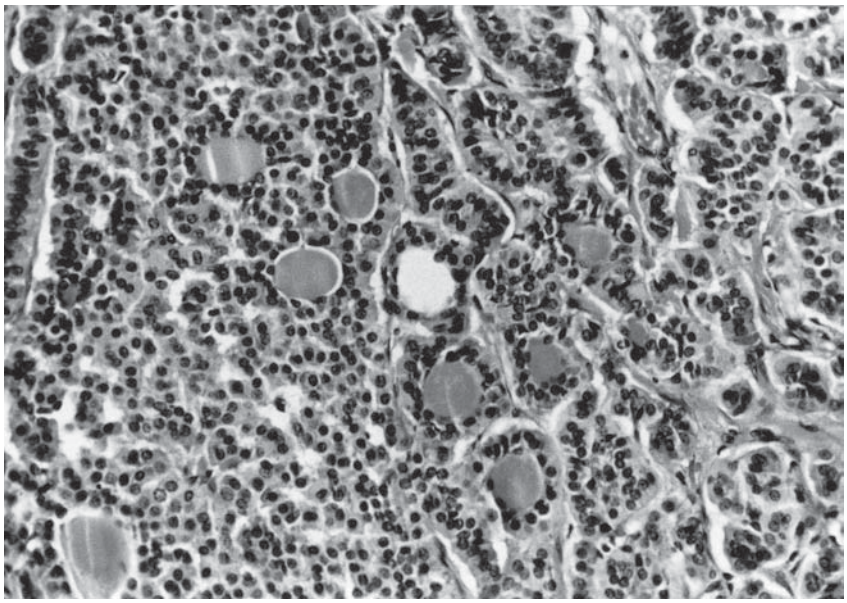

Figure 30 Strumal carcinoid combines the presence of struma ovarii and a neuroendocrine (carcinoid) component.

show a wide variety of microscopic patterns, and it may exhibit foci of hepatic and enteric differentiation. It is usually associated with elevated α-fetoprotein serum levels. Embryonal carcinoma and choriocarcinoma are very infrequent tumors in the ovary in its pure form.

Teratomas are frequent. The vast majority of them are mature cystic teratomas (dermoid cysts), which combine recognizable mature tissues of two or three embryonic layers. In contrast, immature teratomas contain immature tissues. Rarely, mature teratomas may be predominantly or exclusively composed of specific tissues (monodermal teratomas). For example, rare teratomas are exclusively composed of thyroid tissue (struma ovarii) (Fig. 29), which may be functioning, and may even be associated with tyrotoxicosis. Similarly, monodermal teratomas may be exclusively composed of neuroendocrine cells with the typical microscopic pattern of carcinoid tumors of midgut (insular), or foregut or hindgut (trabecular) origin. On rare occasions they may be accompanied by a carcinoid syndrome. Strumal carcinoid is the term used for teratomas combining thyroid tissue and carcinoid elements (Fig. 30). Both struma ovarii and strumal carcinoid may be associated with luteinized stromal cells at the periphery of the tumor [28].

Gonadoblastoma is a rare tumor that arise in dysgenetic gonads. Gonadoblastoma contains both immature germ cells and sex cord stromal elements, with frequent calcification. Most gonadoblastomas are benign, and may even regress; but occasionally may transform into dysgerminoma or other malignant germ cell tumors.

Figure 31 Gross appearance of a "burnout" dysgerminoma in a gonadal streak from a patient with mixed gonadal dysgenesis.

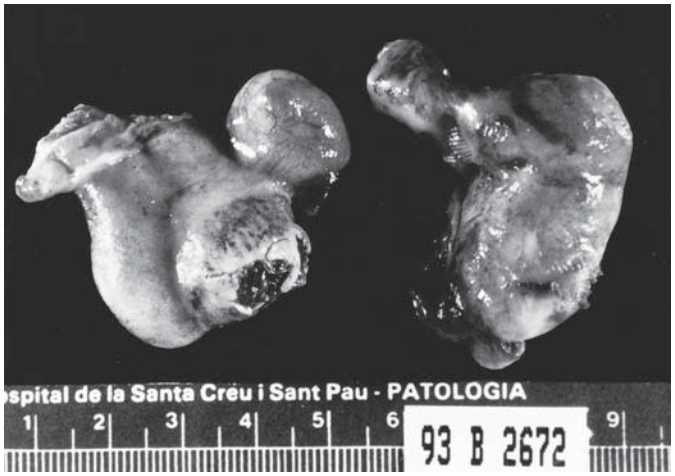

Figure 32 Gross appearance of the abdominal gonads removed from a patient with androgen insensitivity syndrome.

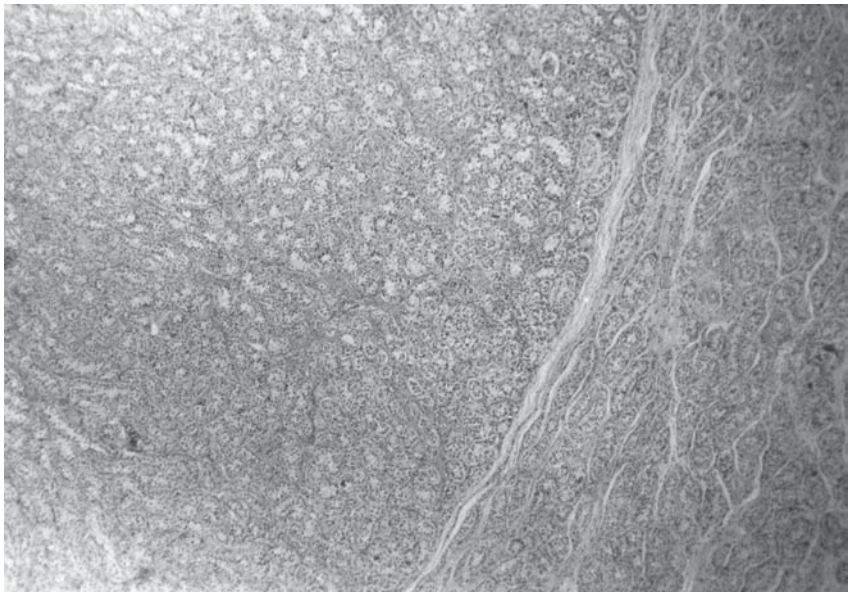

Figure 33 Microscopic appearance of a so-called Sertoli cell adenoma in the gonad from a patient with androgen insensitivity syndrome.

GONADAL DYSGENESIS As mentioned previously, gonadoblastoma and dysgerminoma may arise from gonadal tissue in phenotypic females with pure or mixed gonadal dysgenesis [71,72] (Fig. 31). Patients with pure gonadal dysgenesis (46,XY) have failure of development of the secondary sex organs, and present bilateral gonadal streaks.

Patients with mixed gonadal dysgenesis (45,X; 46,XY mosaicism) show asymmetry of the ambiguous external genitalia and persistent müllerian duct structures. The gonadal constitution consists of either one gonadal streak, and a contralateral gonad more closely resembling a differentiated testis, or bilateral streak gonads. Clusters of Leydig cells may be found in the hilar region of the streaks, and may be responsible for some androgenic manifestations as well as for gradients in testosterone and Δ-$_4$-androstendione concentrations in gonadal blood obtained during surgery.

ANDROGEN INSENSITIVITY SYNDROME Androgen insensitivity syndrome (AIS) is a heterogeneous disorder with wide spectrum clinical manifestations. Patients exhibit a 46,XY karyotype, but express a range of sexual development extending from a predominant male appearance, through incomplete masculinization, to complete testicular feminization (Fig. 32). The molecular abnormality is a defective androgen receptor. In fact, mutations in the androgen receptor gene have been found to be associated with the development of AIS [73]. Patients have bilateral testis, predominantly located in the abdomen. The gonads show immature tubules, containing rare spermatogonia. Prominent Leydig cells and a spindle-cell stroma resembling ovarian stroma are occasionally seen. Hamartomas, Sertoli cell adenomas (Fig. 33), and malignant germ cell tumors are also sometimes detected [74].

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14 Endocrine Testis

Robert E. Emerson, MD, Liang Cheng, MD, and Thomas M. Ulbright, MD

INTRODUCTION

Each major histologic category of testicular tumor includes hormonally functional neoplasms (Table 1). Testicular germ cell tumors, sex cord-stromal tumors, and tumors of neuroendocrine origin all are capable of producing one or more hormones that may result in the clinical presentation of the tumor, be useful for diagnosis of the tumor, or be useful as a tumor marker for guiding treatment or determining prognosis. The endocrine physiology and biochemistry of testicular neoplasms have been reviewed [1–3].

The testis is the major endocrine organ of the male reproductive system. It is composed of several types of cells that are divided between two main anatomic and functional compartments: seminiferous tubules and the interstitial space. Essentially all of the main cell types in the testis have neoplastic counterparts. In contrast to the ovary, where surface epithelial and stromal tumors predominate, germ cell tumors comprise the majority of testicular neoplasms.

Clinical endocrine syndromes associated with testicular tumors can result from several mechanisms. Steroid hormones may be produced directly by the tumor cells. Protein stimulating factors such as gonadotropins may be synthesized that then stimulate secretion of androgens and estrogens. Finally, hormones produced at other sites may be altered within tumor cells, possibly enhancing their effect.

Because this chapter is focused on tumors with endocrine manifestations, the sex cord-stromal neoplasms, which are encountered significantly less frequently than germ cell tumors, are also considered in detail. These tumors are derived from endocrine cells and, consequently, much more frequently demonstrate hormone production. Leydig cell hyperplasia is discussed, as it may histologically simulate neoplasia. Finally, tumors believed to originate from neuroendocrine cells within the testis are considered.

GERM CELL TUMORS

Germ cell tumors comprise the vast majority of primary testicular neoplasms. Preexisting endocrine dysregulation such as hypoandrogenism, prenatal hyperestrogenism, and increased circulating gonadotropin levels may predispose to testicular germ cell neoplasia [4]. The incidence of testicular germ cell neoplasms has been increasing over the past several decades in association with a decline in mean sperm count, and it is possible that the two may be related [5]. The most consistent cytogenetic finding in testicular germ cell tumors is overrepresentation of the short arm of chromosome 12, often in the form of an isochromosome. The significance of this finding to the molecular pathogenesis of these tumors has been reviewed [6]. The majority lack significant endocrine manifestations. Many germ cell tumors, especially nonseminomatous germ cell tumors, do, however, secrete protein products that can be detected in circulating blood. These serum tumor markers are useful in the diagnosis of these tumors and in monitoring response to treatment.

Seminoma generally lacks significant endocrine manifestations [3]. Human chorionic gonadotropin (hCG) is, however, readily demonstrated by immunohistochemistry in the syncytiotrophoblast cells commonly encountered within pure seminoma (Fig. 1) and in some cases, hCG is detectable in cells that are not morphologically distinct from the seminoma cells. There are rare cases of androgen excess due to Leydig cell stimulation by hCG from syncytiotrophoblast cells [7]. Occasionally patients with seminoma and syncytiotrophoblast cells have gynecomastia secondary to peripheral conversion of androgens secreted from hCG-stimulated Leydig cells to estrogens [8]. More rarely, hypercalcemia due to production of a presumed parathyroid hormone-like substance is seen [9,10]. Resolution of hypercalcemia may occur with orchiectomy and retroperitoneal radiotherapy. Lactate dehydrogenase (LDH), a marker elevated in germ cell tumors proportional to the 12p amplification, is elevated in seminoma as well as nonseminomatous germ cell tumors [11]. Serum placental alkaline phosphatase is elevated in about half of patients with seminoma and, in combination with other serum tumor markers, may increase sensitivity of monitoring for seminoma relapse [12].

Clinically the most important serum tumor markers are hCG and α-fetoprotein (AFP). These may be measured by assay of serum from peripheral blood or detected in tissue by immunohistochemistry. Development of radioimmunoassay technology made serum hCG levels useful to monitor disease progression in germ cell tumor patients [13]. Serum AFP is produced consistently by yolk sac tumor elements, and the degree of elevation

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Table 1
Classification of Testicular Tumors with Endocrine Manifestations

Germ cell tumors
Seminoma
Nonseminomatous germ cell tumors
Embryonal carcinoma
Yolk sac tumor
Choriocarcinoma
Teratoma
Mixed germ cell tumor
Sex cord-stromal tumors
Leydig cell tumor
Leydig cell hyperplasia[a]
Testicular tumor of adrenogenital syndrome[a]
Sertoli cell tumor, not otherwise specified
Large cell calcifying Sertoli cell tumor
Granulosa cell tumor
Unclassified and fibroma–thecoma group stromal tumors
Neuroendocrine tumors
Ectopic endocrine tissue[a]
Carcinoid
Paraganglioma
Primitive neuroectodermal tumor
Neuroblastoma
Small cell carcinoma
Syndromes associated with testis tumors
Peutz–Jeghers syndrome
Carney complex
Klinefelter syndrome

[a]These nonneoplastic lesions are included here for the convenience of discussion.

in mixed germ cell tumors correlates with the amount of yolk sac tumor present [14]. The source of hCG is the syncytiotrophoblast cells that are an integral component of choriocarcinoma and that may also occur in seminoma and other germ cell tumors. More recently TRA-1-60 has been studied as a specific marker for embryonal carcinoma [15].

Choriocarcinoma may be associated with several clinical endocrine syndromes (Fig. 2). Gynecomastia is the most common, affecting 10–20% of patients [3]. Production of hCG results in Leydig cell hyperplasia because of its luteinizing hormone (LH)-like activity. The increased androgens then undergo peripheral conversion in adipose tissue to estrogens [16]. Estradiol elevation has also been used to predict tumor recurrence in both seminoma and nonseminomatous germ cell tumors [17]. Also, hCG has thyroid stimulating hormone-like activity, resulting in hyperthyroidism in patients whose germ cell tumors have a choriocarcinoma component [3]. Hyperprolactinemia may rarely be observed [18]. The mechanism of hyperprolactinemia is unclear, but may result from estrogen elevation or stimulation of pituitary lactotrope cells by the free α-subunit of hCG [18]. Apparently pure teratoma may be associated with serum hCG, prolactin, and estrogen elevation, but it is difficult to rule out production of these hormones by undetected foci of choriocarcinoma or another component of a mixed germ cell tumor [3]. It is also well known that pure teratoma of the testis may have nonteratomatous metastases, including choriocarcinoma, that could be responsible for this occurrence.

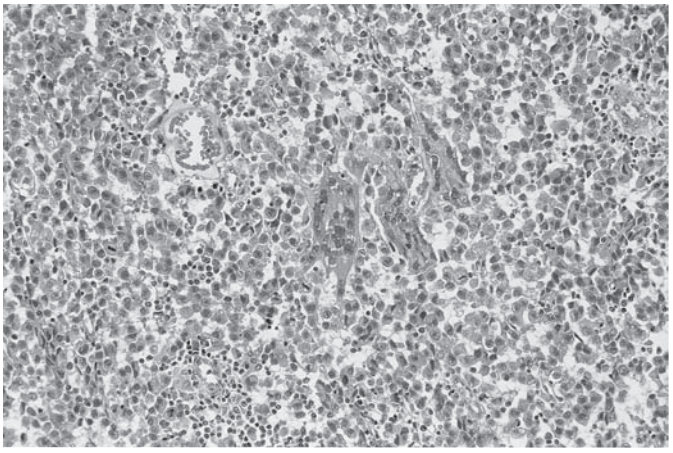

Figure 1 Pure seminoma commonly has occasional syncytiotrophoblast cells, which are not by themselves diagnostic of choriocarcinoma. (Color illustration appears in insert following p. 148.)

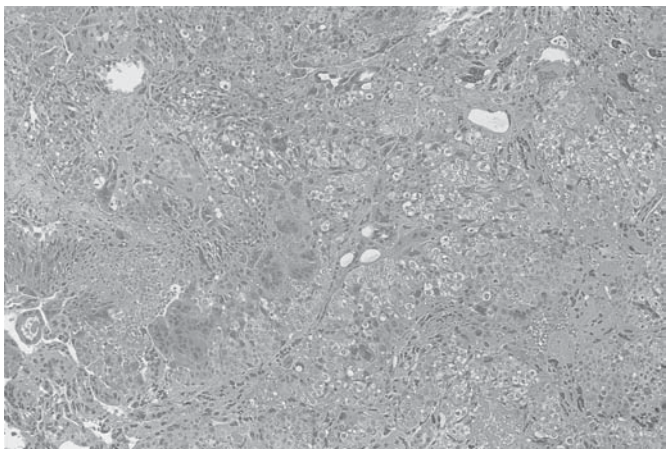

Figure 2 While pure choriocarcinoma is rare, it is a common component of mixed germ cell tumors and can be recognized by the presence of cytotrophoblast cells in association with syncytiotrophoblast cells. (Color illustration appears in insert following p. 148.)

Germ cell tumors may also have other, less-well-understood endocrine manifestations. Spermatogenesis is typically decreased to a greater extent than that expected due to local tumor effect, generalized cancer effect, or treatment effects, and this may result from an underlying endocrine mechanism [19,20] or more likely represent a predisposing feature.

SEX CORD-STROMAL TUMORS

LEYDIG CELL TUMORS Normal Leydig cells are mesenchymal in origin and are arranged as single cells and in small clusters in the testicular interstitium between the seminiferous tubules. They are large and round to polygonal with regular, round, eccentric nuclei. Chromatin is scant and one or two nucleoli may be apparent. Cytoplasmic lipid granules, lipofuscin pigment, and Reinke crystals may be seen. Reinke crystals are refractile eosinophilic cytoplasmic rod-shaped inclusions that are unique to Leydig cells [21].

Leydig cells are the primary site of testicular steroid synthesis. Leydig cells respond to LH, which is produced by the anterior pituitary in response to gonadotropin releasing hormone

(GnRH) production by the hypothalamus. Testosterone provides negative feedback to the pituitary and hypothalamus, inhibiting further LH and GnRH production [22].

Leydig cell tumors comprise 1–3% of primary testicular tumors and are seen in both boys and adult men [22]. In one series, the average age at presentation was 47 yr with a range of 2–90 yr [23]. In patients with Leydig cell tumors, many endocrinologic alterations have been noted [24–26]. Serum testosterone may be increased, normal, or decreased. Serum estradiol, LH, follicle-stimulating hormone (FSH), 17-ketosteroids, and corticosteroids may be normal or increased. Some tumors may produce substances with adrenocorticotrophic hormone (ACTH) activity or FSH-inhibiting activity [24].

Hormonal manifestations, including precocious pseudopuberty and gynecomastia, are usually observed in children with Leydig cell tumors [3]. Approximately 10% of cases of precocious pseudopuberty in boys are the result of Leydig cell tumors. Adult patients are less likely to manifest with endocrine symptoms and present most commonly with an asymptomatic mass [23,27], although approx 15% of adults present because of gynecomastia [23]. If the tumor cells are deficient in one or more enzymes necessary for the production of biologically active steroids, the overproduction may be clinically silent with large quantities of urinary 17-ketosteroids [27]. Less commonly feminization may be seen [3]. Malignant Leydig cell tumors are less likely to have endocrine manifestations, but both androgenic and estrogenic hormonal production can occur [28]. In some cases the steroids secreted by neoplastic or hyperplastic Leydig cells may be more characteristic of adrenal cortex than normal Leydig cells [3]. This may account for some tumors associated with Cushing syndrome believed, incorrectly, to arise from intratesticular adrenal rests [29].

Testicular ultrasound and selective venous sampling with determination of hormone levels may be used to locate clinically occult Leydig cell tumors associated with gynecomastia [30]. Regression of gynecomastia following orchiectomy is the usual outcome [25,26,30,31]. Endocrine testicular function and spermatogenesis may, however, be impaired on a long-term basis [31,32].

Grossly, Leydig cell tumors average 3 cm in greatest dimension, although they may be as large as 10 cm. They are usually nodular, well circumscribed, and typically yellow or yellow-brown [23]. The nodules may be divided by white fibrous bands.

The cells of a Leydig cell tumor are most commonly arranged in solid masses (Fig. 3), although trabeculae, cords, and tubulelike structures may also be seen. The tumor cells are typically large and polygonal with eosinophilic, granular cytoplasm. Reinke crystalloids are seen in 35% of cases on careful examination [23]. Adipose differentiation, ossification, and spindle cell areas may be seen in Leydig cell tumors [33]. Rarely, diffuse spindle cell or frankly sarcomatoid differentiation may occur [33,34]. A microcystic pattern is also unusual and may be confused with yolk sac tumor [35]. Distinguishing the two is critical for clinical management as patients with yolk sac tumor typically receive chemotherapy whereas those with Leydig cell tumors are either followed or have retroperitoneal lymphadenectomy. Negative AFP and placentalike alkaline phosphatase and positive reactions for α-inhibin and Melan A (A103) are expected in Leydig cell tumors in contrast to yolk sac tumors.

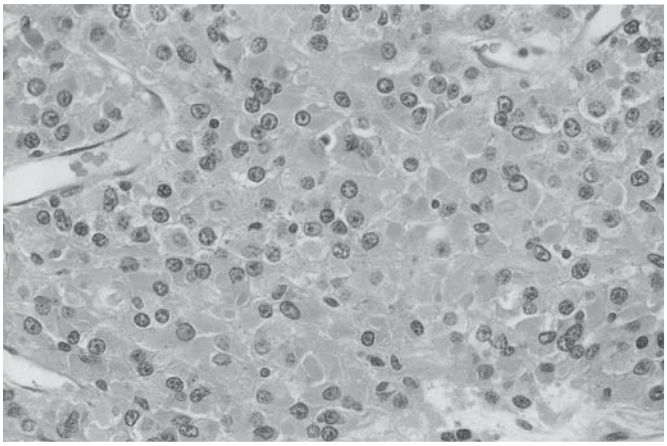

Figure 3 Leydig cell tumors are composed of sheets of polygonal cells that displace and compress the surrounding seminiferous tubules. The nuclei are round, typically with a single prominent nucleolus, and the cytoplasm is eosinophilic. Cytoplasmic Reinke crystals are seen in some cases, with this case showing globular variants of Reinke crystals. (Color illustration appears in insert following p. 148.)

The surrounding testis typically shows decreased spermatogenesis and atrophy of surrounding Leydig cells [26]. Although progressive degeneration of normal Leydig cells surrounding a Leydig cell tumor is probably the typical finding [36], Leydig cell hyperplasia may occasionally be seen [25].

Ultrastructurally, "membranous whorls" have been observed in Leydig cell tumors but not in normal Leydig cells [24]. By immunohistochemistry, Leydig cell tumors are usually positive for vimentin and inhibin while cytokeratin, S100 protein, epithelial membrane antigen, and desmin are less consistently expressed [37,38]. Immunohistochemical staining for A103, an antibody to the Melan-A antigen, is positive in Leydig cell tumors [39]. This stain is also positive in melanoma and adrenal cortical tissue and tumors and, therefore, cannot be used to distinguish hyperplasias from tumors of Leydig cell or adrenal cortical rest origin.

Leydig cell tumors are uniformly benign in children [22]. In adults, approx 10% behave in a malignant manner with retroperitoneal lymph node, pulmonary, and liver metastases being most common [40,41]. Several features correlate with an increased risk of aggressive behavior. These include: older age, absence of endocrine manifestations, large size, infiltrative margins, lymphatic or vascular invasion, necrosis, cellular atypia, and high mitotic rate [23,42,43]. Metastasizing Leydig cell tumors are also more frequently aneuploid and have much higher MIB-1 labeling indices than those that behave in a benign fashion [42,43]. The presence or absence of these features, however, neither guarantees nor excludes the possibility of metastasis [40]. Therefore, benign Leydig cell tumors can be identified unequivocally only by the absence of metastatic tumor on prolonged follow-up, but the features mentioned in the preceding generally allow rational treatment decisions based on very low or high probabilities of metastatic spread.

The most important differential diagnostic considerations are Leydig cell hyperplasia and the "tumors" of the adrenogenital syndrome, which may in fact represent a form of Leydig cell hyperplasia as adrenal rests are not believed to occur within the testis itself [44]. Leydig cell hyperplasia is generally multifocal and bilateral and is seen as multiple small nodules between normal seminiferous tubules. Leydig cell tumor is typically found as a single large mass that compresses the surrounding testis and obliterates the preexisting seminiferous tubules. Marked cytologic atypia, frequent mitotic figures, necrosis, and vascular invasion all indicate a diagnosis of Leydig cell tumor [21]. Although serum ACTH and 17-hydroxyprogesterone elevation are expected in congenital adrenal hyperplasia, one case of Leydig cell tumor that produced both of these hormones has been reported [45]. These hormonal alterations, therefore, while supportive of the diagnosis of tumor of the adrenogenital syndrome, do not exclude a diagnosis of Leydig cell tumor. The often multinodular and bilateral nature of the Leydig cell nodules in congenital adrenal hyperplasia contrast with the solitary, unilateral nature of Leydig cell tumor. In addition, most lesions of congenital adrenal hyperplasia have prominent cytoplasmic lipofuscin that causes the nodules to have a brown to olive green gross appearance and that is readily apparent on microscopic examination as brown cytoplasmic pigment granules.

Inguinal orchiectomy is the treatment of choice in adult men with suspected Leydig cell tumor. In children with suspected Leydig cell tumor, testis-sparing surgery with enucleation of the mass has been proposed as an alternative approach [22]. Retroperitoneal lymphadenectomy is the mainstay of treatment for malignant examples.

LEYDIG CELL HYPERPLASIA Leydig cell hyperplasia (Fig. 4) may be seen in several clinical settings including tuberculosis, syphilis, carcinoma, pernicious anemia, alcoholism, chronic spermatic cord compression, and chronic disease of the bladder and prostate [21]. Congenital Leydig cell hyperplasia may be observed in infants of diabetic mothers, Rh incompatibility, and Beckwith–Wiedemann syndrome [46]. It may also be idiopathic and familial [47], with premature Leydig cell proliferation resulting in male-limited familial pseudoprecocity [48]. This syndrome is gonadotropin independent, unlike the more common central precocious puberty [48,49]. Small Leydig cell nodules often develop in male-limited familial pseudoprecocity [50].

Leydig cell hyperplasia may be seen in congenital adrenal hyperplasia, and some cases previously reported as bilateral Leydig cell tumor of childhood likely are actually hyperplastic in nature [3]. The presence of bilateral testis masses composed of Leydig cells in a boy should put the diagnosis of Leydig cell tumor in doubt and the patient should be evaluated for adrenogenital syndrome which includes bilateral testis enlargement in 80% of cases [22].

Occasionally, Leydig cell hyperplasia may be seen in the interstitium surrounding a Leydig cell tumor [25]. This phenomenon may be explained by production of a stimulating factor by the tumor [25] or by the neoplastic transformation of the cells of preexisting Leydig cell hyperplasia [21]. Leydig cell hyperplasia may also accompany hCG-producing testicular tumors [7]. In these cases large but discontinuous clusters of Leydig cells may be seen surrounding a germ cell tumor that contains syncytiotrophoblast cells.

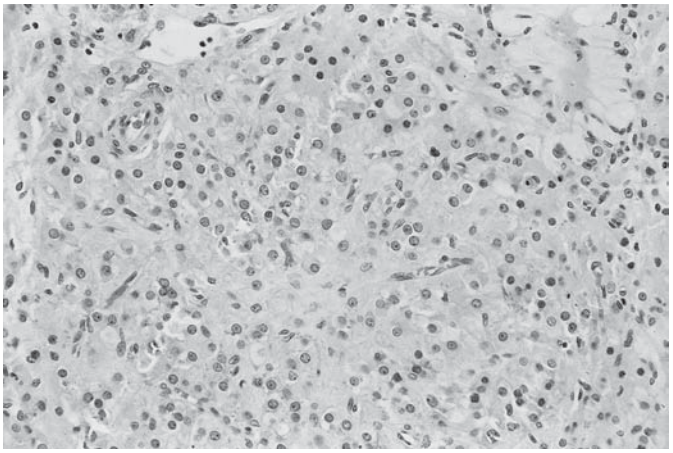

Figure 4 Small to moderate sized nodules arranged between residual tubules are seen in Leydig cell hyperplasia. (Color illustration appears in insert following p. 148.)

Extratesticular Leydig cell rests are common in the scrotum, and these may be confused with extratesticular spread of a testicular Leydig cell tumor or with metastatic tumor from another site [51]. They have the typical cytological features and usually occur as small nests in close association with nerve fibers.

TESTICULAR TUMOR OF THE ADRENOGENITAL SYNDROME Testicular tumors are commonly observed in patients with the adrenogenital syndrome. These tumors arise in response to high levels of ACTH [52,53]. Adrenogenital syndrome results most commonly from 21-α-hydroxylase deficiency with consequent impaired adrenal cortisol synthesis. The resulting increase in ACTH production from the pituitary induces adrenal hyperplasia and production of androgenic steroids with virilizing effects in early childhood. Other enzyme deficiencies may result in milder and often later-presenting forms in which a testis mass is occasionally the presenting finding [22]. Although in the majority of cases the diagnosis of adrenogenital syndrome precedes the detection of a testicular mass, a minority of these cases may first present with a mass [54]. Two thirds of patients with testicular tumors of the adrenogenital syndrome have a salt-losing adrenal disorder [54]. These tumors present at an average age of 23 yr and 83% are bilateral [54]. Androgen excess, in one case resulting in aggressive behavior, has been observed due to these tumors [55]. In one ultrasound study, 16 of 17 patients with adrenogenital syndrome had testicular tumors ranging in size from 0.2 to 4.0 cm [56]. One case was associated with cryptorchidism, seminoma, and testicular myelolipoma [57]. One case of an 8.5 cm malignant Leydig cell tumor arising in congenital adrenal hyperplasia has been reported [58].

Testicular tumors submitted for pathologic evaluation in the adrenogenital syndrome are usually >2 cm, solid, unencapsulated, divided into nodules by fibrous bands, and green-brown in color [54]. They may histologically closely resemble Leydig cell tumors, and can be mistaken for such, possibly resulting in unnecessary orchiectomy [59–61]. The tumor cells are arranged

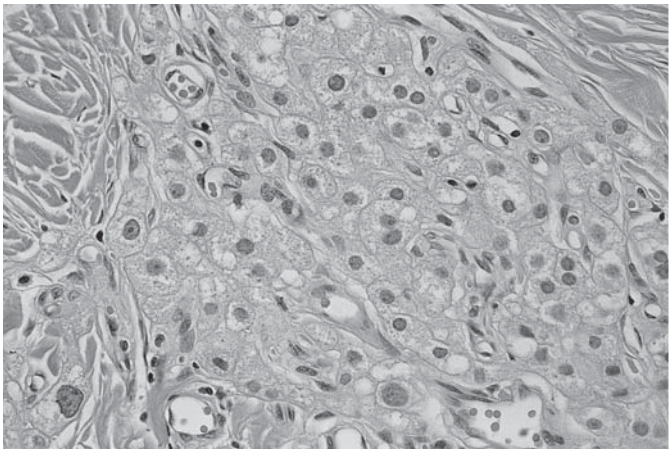

Figure 5 The cells of the testicular tumor of the adrenogenital syndrome closely resemble those of Leydig cell tumor. The history of endocrine abnormalities, bilaterality, and multifocal distribution of tumor is critical for the recognition of this tumor. (Color illustration appears in insert following p. 148.)

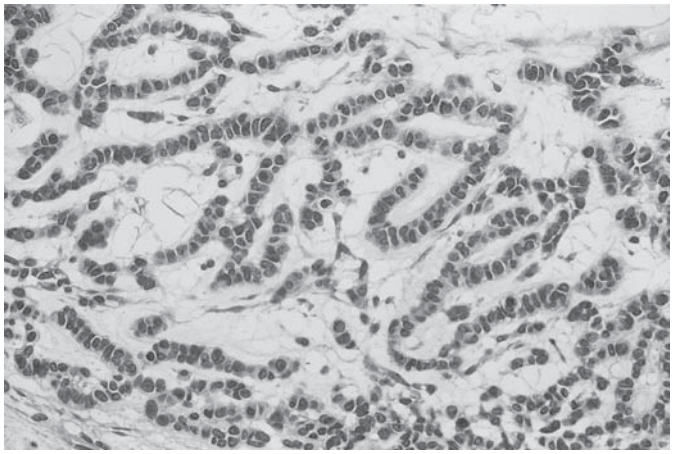

Figure 6 Trabecular architecture is a frequent finding in Sertoli cell tumor. (Color illustration appears in insert following p. 148.)

in nests divided by fibrous stroma (Fig. 5). The cells have abundant eosinophilic cytoplasm with lipochrome pigment but lack Reinke crystals [54]. In the lipoid congenital adrenal hyperplasia variant, lipid vacuole-containing Leydig cells may be seen in the testis outside the tumor, and it is possible that this finding results from defective conversion of cholesterol into pregnenolone [62].

The cell of origin of these "tumors" has been disputed [54]. The cells morphologically closely resemble both Leydig cells and adrenal cortical cells. Evidence cited for Leydig cell origin includes one study of 200 testes that identified paratesticular adrenal cortical rests in 11% of patients, but none within the testis [44]. Against Leydig cell origin are the facts that Reinke crystals are not identified in them [54] and that the tumors develop in a state of ACTH excess and biochemically mimic the function of adrenal cortex [22]. Origin from pluripotential hilus cells has been proposed [54].

The most important differential diagnostic consideration is Leydig cell tumor. Bilaterality and the clinical and endocrinologic manifestations of adrenogenital syndrome provide support for this diagnosis while Leydig cell tumors generally lack these features. The prominent lipofuscin deposits in the cytoplasm and the absence of Reinke crystals are also helpful. Orchiectomy should not be performed in boys with precocious pseudopuberty and a testicular tumor without a complete endocrinologic evaluation that rules out adrenogenital syndrome [63]. These tumors do not require surgical treatment, with regression occurring with steroid replacement therapy [22,64], an observation supporting the belief that they are non-neoplastic nodular hyperplasias.

SERTOLI CELL TUMORS Sertoli cell tumors comprise approx 0.5% of adult and 9% of childhood testicular tumors [65,66]. Gynecomastia and impairment of libido have been reported in association with Sertoli cell tumors, but the association remains controversial owing to the lack of consistent documentation of hyperestrogenism in patients with such tumors [3]. Some cases reported as Sertoli cell tumors may, in fact, represent hyperplastic Sertoli cell nodules.

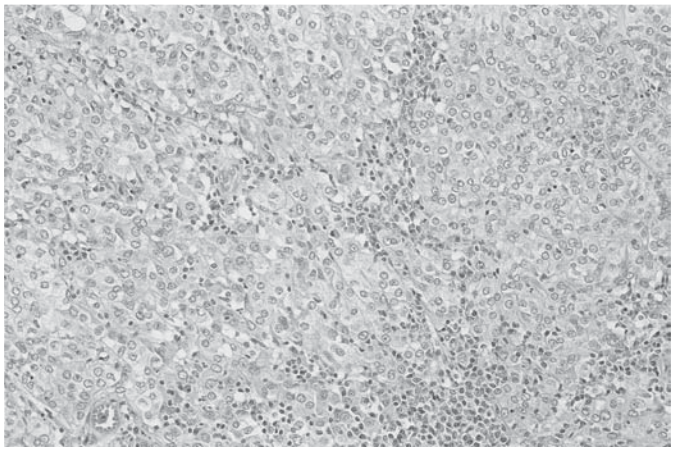

Figure 7 This malignant Sertoli cell tumor has solid architecture, clear cytoplasm, and a lymphocytic infiltrate. Tumors such as this one may be confused with seminoma. (Color illustration appears in insert following p. 148.)

Sertoli cell tumors grossly are well circumscribed, gray to yellow-white, and solid, sometimes with cystic areas [65,67]. Microscopically, solid areas and tubular structures usually are apparent (Fig. 6). The amount of tubular differentiation is variable. The tumor cells have a moderate amount of eosinophilic to clear cytoplasm [67]. Some cases may display extensive sclerosis, and these may be assigned to a specific subtype, sclerosing Sertoli cell tumor [68,69]. Sarcomatoid differentiation with osteosarcomalike foci has been reported in a Sertoli cell tumor [70]. Some malignant cases have a mainly diffuse arrangement of cells with clear cytoplasm and lymphocytic infiltrates that resemble seminoma (Fig. 7), although the lower-grade nuclear morphology and mitotic rate, as well as immunohistochemical staining results help distinguish the two [71].

Sertoli cell tumors are positive for vimentin and, in some cases, for keratin, epithelial membrane antigen, and smooth mus-

cle actin as well [66,71]. Inhibin marks sex cord-stromal tumors, including Sertoli cell tumors [38], and S100 protein immunostaining may also be seen in some cases [37]. Placental alkaline phosphatase is negative, in contrast to seminoma, which also shows negative reactivities for epithelial membrane antigen, α-inhibin, and AE1/AE3 cytokeratin.

Although the majority of adult Sertoli cell tumors are benign, approx 10% have been associated with metastases [65]. In 18 cases of pediatric Sertoli cell tumors, no recurrences were observed following orchiectomy [66], but the high proportion of infants in that series has raised the question that a number of these cases were juvenile granulosa cell tumors. There are clearly occasional malignant cases in children, in contrast to the experience with childhood Leydig cell tumors.

LARGE CELL CALCIFYING SERTOLI CELL TUMOR Large cell calcifying Sertoli cell tumor is a rare tumor of Sertoli cell origin that can result in hyperestrogenemia and gynecomastia [72]. Less commonly, testosterone production may be observed [73]. When this group of tumors was first described, it was noted that they were commonly associated with bilaterality, multifocality, endocrine disorders, familial occurrence, and cardiac myxomas [74]. Later it was determined that these associations were due to their occurrence as a part of Peutz–Jeghers syndrome and Carney complex.

While large cell calcifying Sertoli cell tumor is characteristic of Peutz–Jeghers syndrome and Carney complex [75], it may also be seen in patients who are not affected by either of these disorders [76]. Those cases not associated with one of these syndromes usually present between 13 and 34 yr of age, are more likely to be unilateral and unifocal, and can be treated with orchiectomy alone as the prognosis is generally favorable [76–78], unless features associated with malignant behavior are identified [79]. One case of large cell calcifying Sertoli cell tumor was associated with unilateral renal agenesis and inferior vena cava duplication, but this may be a chance association [77].

Grossly the tumors average approx 2 cm at presentation and are well circumscribed, solid, and yellow to gray-tan [72,76]. The tumor cells are arranged in nests or cords surrounded by basement membrane material and myxoid to fibrous stroma with an inflammatory infiltrate that includes neutrophils [76]. The tumor cells are large with lightly to densely eosinophilic cytoplasm [72]. Centrally located small spherical or larger amorphous calcifications are typical but not always present (Fig. 8) [76]. An interesting finding in some cases is the presence of α-1-antitrypsin-containing cytoplasmic hyaline globules in Sertoli cells in the uninvolved seminiferous tubules [76].

Charcot–Böttcher crystalloids, unique to Sertoli cells, may be seen by electron microscopy as ellipsoidal aggregates of filaments [72,73]. Tumor nests are surrounded by a basal lamina [80]. Cytoplasmic dense granules, lipid droplets, and intracytoplasmic lumina with microvilli may be seen [80]. Strong vimentin and focal keratin staining have been noted by immunohistochemistry [76]. Staining for S100 protein may be positive [80]. Inhibin is expressed by Sertoli cells and may be useful as a serum tumor marker [81].

These tumors generally behave in a benign fashion [78]; however, occasional cases are malignant [79,82,83]. Malignant tumors may have endocrine manifestations including gynecomastia [83]. A study comparing the clinical and histologic features of benign and malignant large cell calcifying Sertoli cell tumors found that malignant tumors presented at a higher mean age (39 yr vs 17 yr), were less likely to be bilateral or multifocal, were less likely to be associated with Carney complex or endocrine manifestations, were more likely to be >4 cm, and were microscopically more likely to show necrosis, cellular atypia, high mitotic rate (more than three per 10 HPF), and vascular invasion [79].

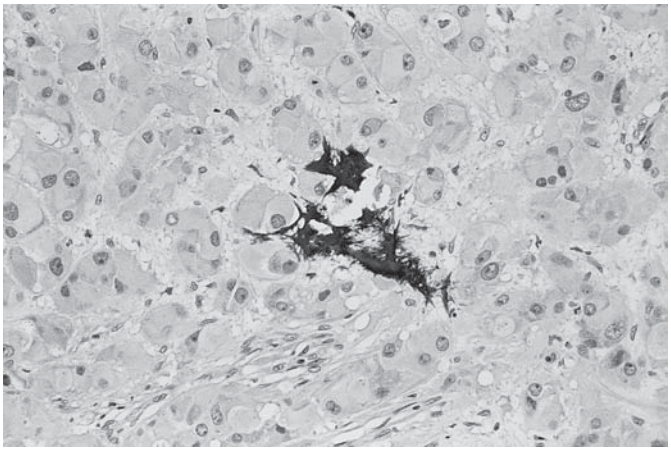

Figure 8 Cells with abundant eosinophilic cytoplasm and foci of calcification characterize large cell calcifying Sertoli cell tumor. (Color illustration appears in insert following p. 148.)

GRANULOSA CELL TUMOR Granulosa cell tumors very rarely occur in the testis. These tumors morphologically resemble the more common ovarian granulosa cell tumor and the granulosa cells of the normal ovarian follicle. The presence of this tumor in the testis has been explained by the capacity of primitive sex-cord cells to differentiate into tissue found in either type of gonad [84]. As in the ovary, two types of granulosa cell tumor are recognized: adult type and juvenile type. The adult type has been reported in men from 16 to 76 yr of age [85]. Adult-type granulosa cell tumors may be associated with hyperestrogenemia and resulting gynecomastia and loss of libido [3]. However, these tumors more commonly lack endocrine manifestations [85].

The adult-type granulosa cell tumors are grossly well circumscribed and usually yellow. Solid, cystic, microfollicular, and trabecular patterns may be seen on microscopic examination. Call–Exner bodies, rosettelike structures with a round, smooth-bordered central space, are seen in about half of the cases (Fig. 9). The tumor cells have scant cytoplasm and oval nuclei. A key cytologic finding is the presence of longitudinal nuclear grooves [85]. The tumor cells are immunohistochemically positive for vimentin and negative for epithelial membrane antigen [85]. Focal cytokeratin positivity has been reported [86], but not in all studies [85,87]. Membranous O13 (CD99, Ewing sarcoma antibody) positivity has also been noted [86]. Ultrastructural findings include occasional desmosomes, cytoplasmic glycogen, rough endoplasmic reticulum, and folded nuclear membranes, similar to ovarian granulosa cell tumors [88].

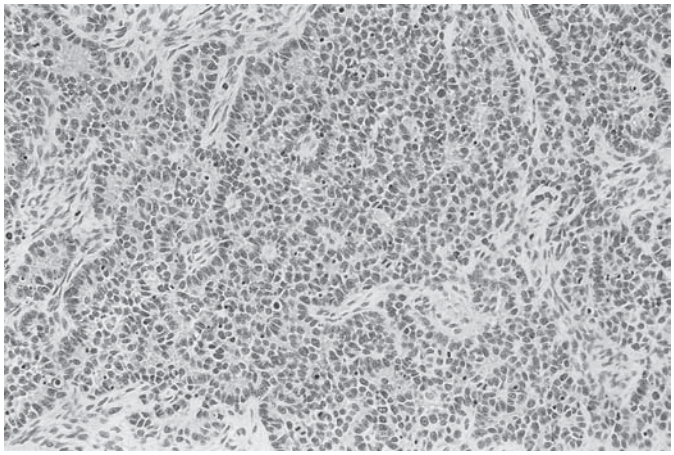

Figure 9 Adult type granulosa cell tumor is composed of sheets of cells with pale cytoplasm and rosettelike Call–Exner bodies. At higher power longitudinal nuclear grooves are seen in the oval nuclei. (Color illustration appears in insert following p. 148.)

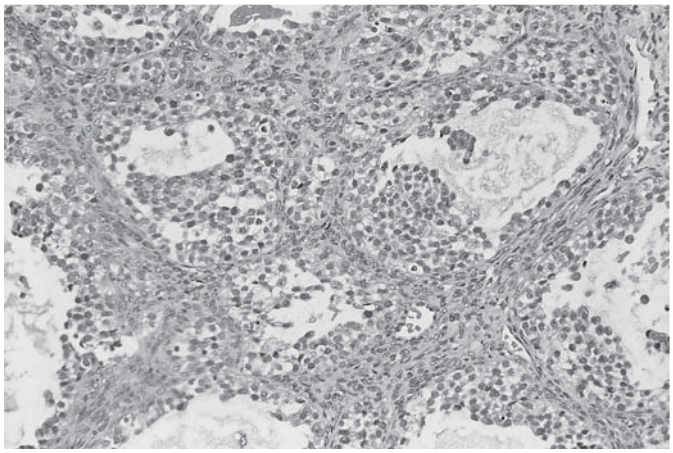

Figure 10 In juvenile type granulosa cell tumor, folliclelike structures or cystic change may be seen. The nuclear grooves typical of adult type granulosa cell tumor are not typically seen. (Color illustration appears in insert following p. 148.)

Retroperitoneal or other metastases have been documented in 4 of 19 cases (21%) reported or reviewed in one study [85]. Clinical and histologic findings do not reliably predict behavior in this group of tumors, but large size (>7 cm), lymphovascular invasion, and necrosis are seen more frequently in tumors that metastasize [85]. In one case, survival for 14 yr with no recurrence followed orchiectomy, retroperitoneal lymphadenectomy, and radiation therapy in a patient with retroperitoneal lymph node metastases [89].

Juvenile granulosa cell tumors may be congenital or they may present in infants, usually at <6 mo of age [90]. They compose approx 6% of childhood testicular tumors [66]. They may be associated with testicular torsion [91]. In many cases they are associated with ambiguous external genitalia, an abnormal somatic karyotype, or mixed gonadal dysgenesis [92,93]. The somatic karyotype typically shows Y chromosome abnormalities such as an extra, ring, or isochromosome.

These tumors are up to 5 cm and may be cystic or solid [66]. Occasionally extensive cystic change may be seen [93]. Histologically they are composed of cells arranged in nodules and that focally form folliclelike structures with central lumens having mucin-containing fluid (Fig. 10). The cells have abundant eosinophilic cytoplasm and oval nuclei. The grooves typical of adult type granulosa cell tumor usually are not seen. The tumors are positive for vimentin and inhibin by immunohistochemistry and some cases may also be positive for smooth muscle actin or cytokeratin [66,94,95]. Ultrastructurally, muscle-like filaments with dense bodies are apparent, suggesting dual epithelial and mesenchymal differentiation [94].

In three series totaling 32 cases, no recurrences were noted following orchiectomy [66,90,94].

UNCLASSIFIED STROMAL TUMORS AND STROMAL TUMORS OF THE FIBROMA–THECOMA GROUP These very rare, generally benign tumors have been the subject of single case reports or small series [96–100]. Actin positivity in some cases suggests origin from peritubular myoid cells [97,98,100]. As endocrinologic manifestations have not been reported, these tumors will not be considered further here.

NEUROENDOCRINE TUMORS

ECTOPIC ENDOCRINE TISSUE Ectopic adrenal cortical tissue may be found adjacent to, but not within, the testis [44]. Adrenal cortical hormone–producing tumors arising within the testis probably represent adrenal cortex-like differentiation in Leydig cell tumor [3]. Hyperplasia of paratesticular adrenal cortical rest tissue may be seen in Nelson's syndrome (growth of an ACTH-producing pituitary adenoma and cutaneous hyperpigmentation following bilateral adrenalectomy) [101] and in congenital adrenal hyperplasia.

CARCINOID TUMORS Primary carcinoid tumors of the testis comprise 0.23% of testicular tumors and they typically are not associated with the carcinoid syndrome [102,103]. They commonly present at an older age than germ cell tumors, with a mean of 43 yr in one series of 10 cases [102]. Single cases have been reported in a 10-yr-old child [104] and a 19-yr-old man [105]. A single case associated with an undescended testis [106] and a single bilateral case [107] have been reported. Serum AFP, hCG, and serotonin are generally not elevated [102,108]. Ultrasound demonstrates a solid mass with calcifications, but this finding is not specific [109,110]. A report of one case describes carcinoid syndrome with diarrhea and postprandial sweating at the time of presentation with a testicular mass [111]. A second patient had watery diarrhea that resolved following orchiectomy. This case was associated with an elevated serum serotonin level [112]. Facial flushing and sweating in the absence of diarrhea have also been reported [104, 113]. One case was reported in association with peptic ulcer disease, but the association may be coincidental [114].

Histologically, they closely resemble midgut insular carcinoid tumors. The tumor cells are arranged in interconnecting nests, acini, and solid areas (Fig. 11). The cells are round to polygonal with finely granular cytoplasm and round, relatively uniform nuclei with patchy chromatin condensation. Ultrastructurally, pleomorphic granules similar to those observed in midgut carcinoids may be observed [115].

The tumor cells are argentaffin and argyrophil positive and periodic acid-Schiff positivity may be noted in the acinar struc-

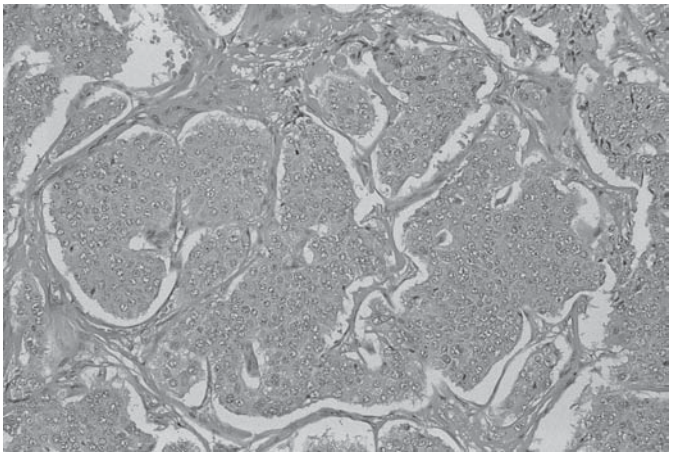

Figure 11 Carcinoid tumors of the testis resemble midgut carcinoid tumors with interconnecting nests of tumor. The cells have neuroendocrine nuclear features and finely granular eosinophilic cytoplasm. (Color illustration appears in insert following p. 148.)

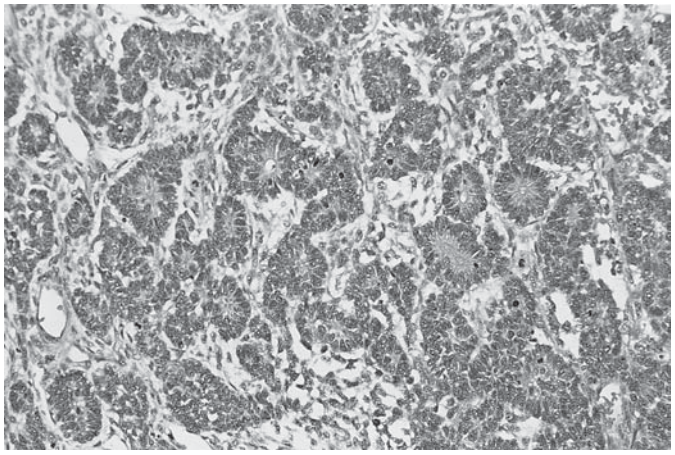

Figure 12 Primitive neuroectodermal tumors are composed of cells with scant cytoplasm and crowded nuclei with fine chromatin. Rosette-like structures are common. (Color illustration appears in insert following p. 148.)

tures. Immunohistochemical staining for low molecular weight cytokeratin, neuron-specific enolase, chromogranin, and synaptophysin are positive [108,116,117]. Serotonin, substance P, gastrin, and vasoactive intestinal peptide immunoreactivity may also be demonstrated [117,118].

The primary clinical and pathologic considerations following a testicular carcinoid tumor diagnosis is exclusion of the possibility of metastasis from another site and exclusion of an associated germ cell tumor component [119,120]. Proposed criteria for distinction from metastasis are unilateral tumor, absence of tumor involving the lung and gastrointestinal tract, negative urine serotonin following surgery, and absence of carcinoid tumor involving other sites on follow-up [121]. Octreotide scintigraphy may be used to exclude extratesticular carcinoid tumors [122]. Metastatic carcinoid originating from the ileum may present as a mass within [102] or adjacent to [123, 124] the testis. Secondary carcinoid tumor is typically observed in the setting of widespread metastasis and, in contrast to primary carcinoid, is commonly associated with urinary serotonin metabolite elevation [102]. Carcinoid tumors metastatic to the testis have a very poor prognosis while primary testicular carcinoid tumors have an excellent prognosis and are generally cured by orchiectomy [125,126]. Inguinal orchiectomy alone is adequate treatment [103]. Occasionally carcinoid of the testis may demonstrate metastasis. One case report described widespread metastasis at the time of orchiectomy [127] while another reported metastatic spread with associated carcinoid syndrome 17 yr following orchiectomy [128]. In one series, 2 of 10 cases progressed to metastatic disease [102].

Although the majority of testicular carcinoid tumors are unassociated with teratoma, they may also arise as a component of mature teratoma [102,129–131]. Enteroendocrine cells have been identified in up to 21% of testicular teratomas [132,133]. These tumors have not been associated with carcinoid syndrome and they do not appear to alter the prognosis of primary testicular teratoma. Metastasis of the carcinoid component to a preaortic lymph node has been observed [134].

PARAGANGLIOMA Paraganglioma of the spermatic cord, but not within the testis, has been the subject of single case reports [135,136]. Histologically it appears as nests of polygonal cells in a "Zellballen" pattern. The cells have abundant eosinophilic granular cytoplasm. The tumor cells are immunohistochemically positive for neuron-specific enolase, chromogranin A, and synaptophysin. Sustentacular cells surrounding the nests are positive for S100 protein. These tumors have not been associated with clinically significant hormonal manifestations.

PRIMITIVE NEUROECTODERMAL TUMOR Primitive neuroectodermal tumor (PNET) has been described arising in testicular germ cell tumors [137,138]. One report of 29 cases of PNET arising in a testicular germ cell neoplasm details the use of immunohistochemistry to subclassify these tumors further into neuroblastoma, medulloepithelioma, peripheral neuroepithelioma, and ependymoblastoma [139]. The PNET component is composed of small round blue cells and may be seen in the primary tumor, metastases, or both (Fig. 12). In this study, PNET limited to the testis did not adversely affect survival but PNET in a metastasis had a very poor prognosis as this component is resistant to germ cell tumor chemotherapy.

NEUROBLASTOMA In a series of 11 cases of neuroblastoma involving the testis, all proved to be metastatic on further evaluation [140]. One case of congenital paratesticular neuroblastoma likewise subsequently proved to be secondary from an adrenal primary [141]. A single case of primary testicular neuroectodermal tumor compatible with neuroblastoma has been reported [142]. No other germ cell tumor component was identified in association with this tumor, but it is possible that the tumor arose from a germ cell neoplasm that was not identified or subsequently regressed.

SMALL CELL CARCINOMA Small cell carcinoma has not been reported as a primary tumor arising in the testis. Small cell carcinoma metastases from a lung primary tumor have occasionally been reported [143,144]. These have lacked paraneoplastic and endocrine manifestations.

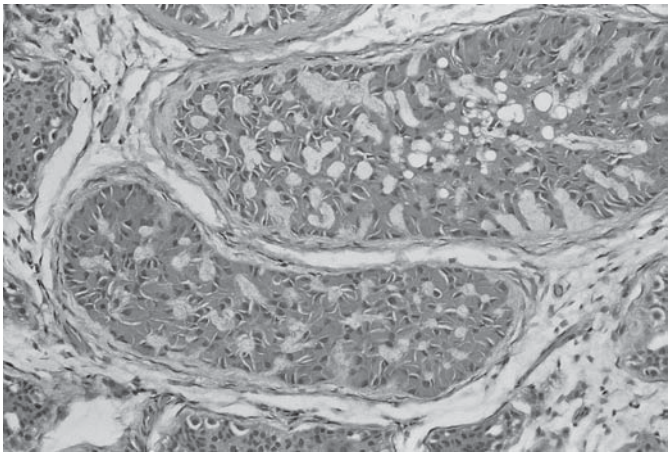

Figure 13 Intratubular Sertoli cell proliferation may be seen in patients with Peutz–Jeghers syndrome. (Color illustration appears in insert following p. 148.)

SYNDROMES

PEUTZ–JEGHERS SYNDROME Peutz–Jeghers syndrome is an inherited multiple neoplasia syndrome in which patients develop mucocutaneous pigmentation, gastrointestinal polyposis, and numerous other tumors. The syndrome usually results from a chromosome 19p mutation of the *STK11/LKB1* gene [75]. Several cases of Sertoli cell tumors in boys with Peutz–Jeghers syndrome have been reported [145–147]. These tumors may be bilateral and multicentric and are associated with gynecomastia, rapid growth, and advanced bone age [145,148]. These cases have been classified as the large cell calcifying Sertoli cell tumor subtype of Sertoli cell tumor, a subtype characteristic of Peutz–Jeghers syndrome and Carney complex [75]. Intratubular Sertoli cell proliferations may also be seen (Fig. 13) [149]. Leydig cell tumors may also be seen [75].

CARNEY COMPLEX Carney complex is an autosomal dominant multiple neoplasia syndrome that includes primary pigmented nodular adrenocortical disease, pituitary adenomas, thyroid adenomas or carcinomas, cardiac myxomas, gastric leiomyosarcoma, ovarian cysts, and other tumors. The complex results from, in most cases, a 2p or 17q mutation [75]. Testicular manifestations are present in more than half of male Carney complex patients [75]. The most common testicular tumor, seen in one third of male patients, is large cell calcifying Sertoli cell tumor [150]. This tumor may be accompanied by symptomatic hormone production with gynecomastia in prepubertal boys [150,151]. Other endocrine manifestations may result from synchronous tumors at other sites, such as Cushing's syndrome due to an adrenal adenoma [151]. Other less frequent tumors associated with Carney complex include Leydig cell tumors, seen in two patients, and paratesticular pigmented adrenal cortical rest tumors, seen in three patients [150].

KLINEFELTER SYNDROME The incidence of testicular neoplasia is increased in several disorders of sexual differentiation. Mediastinal germ cell tumors may occur in patients with Klinefelter syndrome and, more rarely, testicular germ cell tumors may also be seen [152]. Cryptorchidism, but not the increased gonadotropin levels associated with Klinefelter syndrome, is believed to be associated with testicular neoplasia [153]. The tumors seen in cryptorchidism, XY gonadal dysgenesis, and testicular feminization are generally germ cell tumors or gonadoblastomas and typically lack endocrine manifestations [153].

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15 Endocrine Tumors of the Gastrointestinal System

GUIDO RINDI, MD, PHD, SILVIA PIZZI, PHD, TIZIANA D'ADDA, BSCD, AND CESARE BORDI, MD

INTRODUCTION AND HISTORICAL BACKGROUND

The endocrine tumors of the gastrointestinal tract are reputed to originate from cells belonging to the so-called diffuse endocrine system (DES) of the gut. The history of such enteroendocrine cells and of derived tumors begins with the early development of histology and histochemistry.

Peculiar cells of the gastric [1] and intestinal mucosa [2–4] attracted the attention of scientists as early as the second half of the 19th and early 20th centuries. Because their staining was attributed to interaction with chromium salts [4], these cells were named enterochromaffin cells [5]. The concurrent discovery of secretin by Bayliss and Starling in 1902 [6,7] proved the gut as the source of bloodborne agents, "hormones," capable of eliciting physiological effects at a distance. In 1938, Feyrter described epithelial cells in different organs of the human body that failed to take up conventional stains [8]. These cells were named "clear cells" and included those with intrinsic silver-reducing power (chromaffin cells) shown by Masson [9]. It was suggested that these cells had local, "paracrine" action via production and secretion of peptides or amines and, because of their wide distribution, they were grouped as the so-called DES [10]. More recently, in 1966, Pearse identified a group of cells containing amines and/or with the property of taking up amine precursors that are then transformed into amines by intracellular decarboxylation [11]. These cells, largely corresponding to the "clear cells" of Feyrter, were grouped in the amine uptake and decarboxylation (APUD) system [12], which comprised, together with other types, the argentaffin [9], 5-hydroxytryptamine-storing [13] cells of the gastrointestinal tract.

In parallel with the development of the concept of the DES, a nonconventional, slow-growing epithelial tumor was identified and defined as "karzinoide" (carcinoid, i.e., carcinomalike) by Öberendorfer [14]. The argentaffin properties of some of these tumors were described by Gosset and Masson (1914) [15] and their relationship with the enterochromaffin cells was subsequently established [16].

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HISTOGENESIS

Since the initial observation of Bayliss and Starling, a large number of hormones was identified in the gut, so that the gastroenteropancreatic tract is now recognized as the largest endocrine organ of the human body [17]. The gut DES is remarkably heterogeneous and is composed of as many as 15 highly specialized epithelial cells of endodermal origin [18]. Gut cells of the DES constitute a complex regulatory network whose function includes the fine tuning of secretion, absorption, motility, cell proliferation, and possibly immune-barrier control. Such functional activities are exerted by synthesis and release of peptide hormones and biogenic amines specific for the individual cell types.

Gut cells of the DES share with neural cells a number of antigens, commonly defined as "neuroendocrine markers" [19], a finding justifying the term "neuroendocrine" commonly used to connote DES cells and their tumors. For a general assessment of their neuroendocrine profile the first methods developed and still in use include silver impregnation techniques, such as the Masson–Fontana stain (Fig. 1A) demonstrating argentaffinity and Grimelius' stain (Fig. 1B) demonstrating argyrophilia, that is, the ability of endocrine cells to take up and reduce silver ions in the absence (argentaffinity) or in the presence of reducing agents (argyrophilia) [20–22].

Such techniques, although effective and reproducible, have now been largely substituted by immunohistochemistry for cytosol markers such as neuron-specific enolase (NSE) and protein gene product 9.5 (PGP 9.5) [23–26], for granular markers associated with electron-dense granules or large-dense-core vesicles (LDCV) such as chromogranins and related fragments and protein 7B2 [27–30], or for small synaptic-like vescicle (SSV) markers such as synaptophysin [31–33]. More recently, two isoforms of an ATP-dependent vesicular monoamine transporter protein (VMAT1 and VMAT2) have been identified [34]. Both isoforms are found in the adrenal medulla, but VMAT1 alone is expressed in gut enterochromaffin (EC) cells and VMAT2 alone

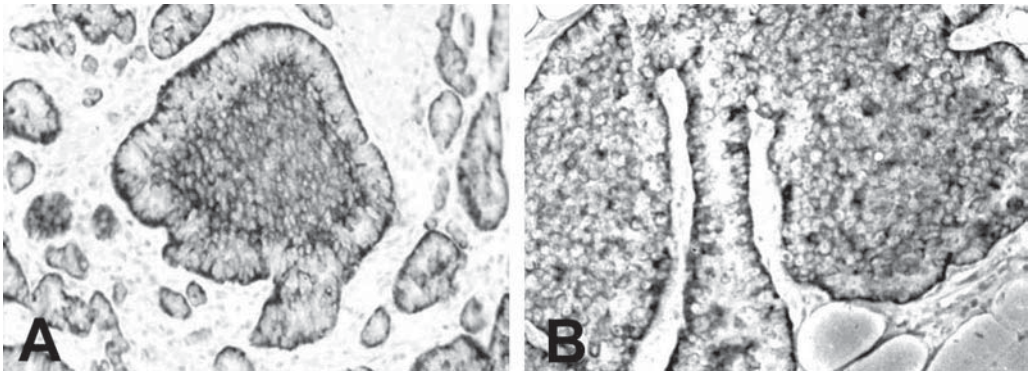

Figure 1 Intense argentaffinity (**A**) and argyrophilia in (**B**) EC cell tumors of the small intestine; note the islet structure with silver accumulation at the periphery of cells. Masson–Fontana (**A**) and Grimelius (**B**) silver impregnation methods.

Table 1
Distribution and Major Features of the Endocrine Tumors of the Gastrointestinal Tract

			Stomach		*Intestine*						
					Small				*Large*		
Tumor type	*Main cell type*	*Main product*	*CF*	*An*	*D*	*J*	*I*	*Ap*	*C*	*R*	*Possible syndrome*
Well	D	Somatostatin			+	+					Somatostatinoma
differentiated	EC	5-HT	+	+	+	+	+	+	+	+	"Carcinoid"
	ECL	Histamine	+								"Atypical carcinoid"
	G	Gastrin		+	+	+	+				ZES
	L	PYY/eg			+	+	+	+	+	+	–
Poorly differentiated	s/i cells	–	+	+	+	+	+		+	+	–

Modified from Rindi et al. 2000 [111].

CF, Corpus-fundus; An, antrum; D, duodenum; J, jejunum; I, ileum; Ap, appendix; C, colon; R, rectum; +, presence of tumor; EC, enterochromaffin cell; ECL, enterochromaffinlike cell; 5-HT, 5-hydroxytryptamine (serotonin); ZES, Zollinger–Ellison syndrome; PYY/eg, peptide tyrosine-tyrosine/enteroglucagon; –, not defined; s/i, small–intermediate-sized cells.

in the gastric enterochromaffinlike (ECL) cells as well in pancreatic islets and derived tumors [35–37].

The above markers allow the assessment of the neuroendocrine nature of the cells under study and are thus defined as "general markers." The full identification of their endocrine cell product(s) is achieved either by electron microscopy or by immunohistochemistry for specific hormones/amines, thus defined as "specific markers" [18].

In addition to these features, gut endocrine cells and derived tumors are characterized by the production of several growth factors and by the expression of their relevant receptors [38]. This property may possibly explain some clinical manifestation of hyperfunctional syndromes associated with endocrine tumors (e.g., carcinoid heart disease) [39] and some histological aspects (e.g., smooth muscle cell proliferation, as often observed in gastric tumors) [38]. Finally, gut endocrine cells and derived tumors diffusely and abundantly express the somatostatin receptor subtype 2 (SSR2) [40–44]. The latter feature is of paramount importance for both diagnostic and therapeutic applications [45,46].

Considering the anatomical and functional heterogeneity of DES cells, it is no wonder that gut endocrine tumors constitute a heterogeneous group with remarkable differences regarding genetic background, functional properties, related clinical syndromes, clinicopathological association, and prognosis (Table 1).

GENETIC BACKGROUND

In general, the endocrine tumors of the gastrointestinal tract have been poorly studied. The tumors investigated are relatively few, frequently lack tumor cell type details, and, finally, most investigations lack mutational analysis. These facts reflect the paucity of proper, fresh frozen material available for study. In the following paragraphs some of the evidence available to date is briefly described (for details see [47]).

A major step for understanding the genetic basis of gastrointestinal endocrine tumors was the identification of the gene cosegregating with the multiple endocrine neoplasia type 1 (MEN 1) syndrome, in which endocrine tumors of the pancreas, duodenum, and stomach are found [48]. Located on chromosome 11q13, the *MEN1* gene is found to act as a tumor suppressor gene [49]. In familial forms the tumor genotype consists of an inherited germline mutation of the *MEN1* gene, with somatic loss of function of the wild-type allele either following

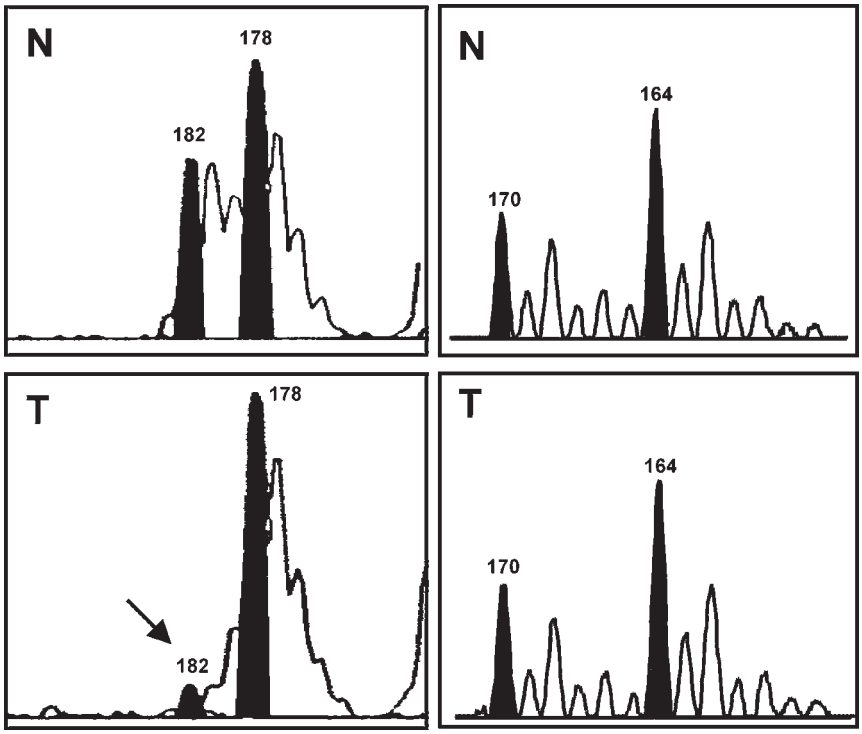

Figure 2 (**Left**) Allelic loss at the 11q13/*MEN1* locus in a type I gastric carcinoid found after PCR amplification of the microsatellite marker *PYGM* with almost complete disappearance of the peak corresponding to the larger allele (*arrow*) in the tumor tissue (T) as compared to normal tissue (N). (**Right**) Absence of allelic loss at the same microsatellite marker in a midgut (ileal) carcinoid.

chromosomal deletion (loss of heterozygosity [LOH]) or point mutations. The *MEN1* gene spans 9 kb and consists of 10 exons with an 1830-bp coding region encoding a novel 610 amino acid protein, referred to as menin [50]. Menin localizes to the nucleus and inhibits the AP-1 transcriptional factor JunD [51]. Moreover, menin interacts with NF-κB proteins, inhibiting NF-κB-mediated transcriptional activation [52].

Available data point to a significant difference between tumors of the region deriving from the primitive foregut (stomach, duodenum, and upper jejunum) and those of midgut and hindgut derivatives. Such a difference reflects the tumor involvement in MEN 1 syndrome with tumor growths restricted to foregut regions, mostly ECL cell tumors of the stomach and duodenal, functioning, G-cell tumors (gastrinomas). Indeed, well-differentiated gut endocrine tumors often display *MEN1* gene LOH in both the stomach [53–57] and the upper intestine [54,56, 58–61]. Gastric endocrine tumors with *MEN1* gene abnormality included poorly differentiated carcinomas (see the following paragraph for tumor definitions) [57,62]. Functioning G-cell tumors of the duodenum and relative metastasis composed the largest fraction of investigated intestinal tumors. Notably, the 11q deletions reported in upper gut endocrine tumors (Fig. 2A) may display continuous losses up to the most distal marker investigated [57], as also seen in pancreatic endocrine tumors [63] (Table 2). Mutations of the *MEN1* gene were also reported in a fraction (approx 30%) of sporadic tumors of the upper gut [60,61,64–66]. In addition, *MEN1* gene mutations were reported in one poorly differentiated endocrine carcinoma of the stomach [62]. This evidence strongly suggests a significant role of the *MEN1* gene, possibly as a tumor-inducing defect in endocrine cells of the upper gut.

In contrast, relatively few tumor cases of the midgut and lower gut have been investigated either for *MEN1* allelic loss or mutation resulting mostly negative for both analyses (Fig. 2B) [47]. This was confirmed by a recent study showing an overall 9% LOH rate of 11q markers investigated in 16 ileal, 6 appendicular, and 3 rectal well-differentiated endocrine tumors [63]. Notably, in this report 11q losses were of limited extension, if not interstitial, and consistently discontinuous (Table 2). *MEN1* gene mutation was found only in one of 12 midgut endocrine tumors so far investigated [64,65]. This evidence would not support a significant role of the *MEN1* gene in midgut and lower gut endocrine tumors.

Other genes have been investigated, although information is again mostly scant and incomplete. In the stomach the *RegIalfa* gene located on chromosome 2p and involved in the functional control of ECL cell growth proved mutated in well-differentiated endocrine tumors [67].

Extensive X-chromosome allelic deletions were found in all malignant gastric endocrine carcinomas investigated ($n = 4$), whereas no significant loss was reported in benign tumors ($n = 29$) [57]. In keeping with similar data obtained in pancreatic endocrine tumors [68,69], such evidence supports an association between X-chromosome LOH and malignancy as limited to foregut endocrine tumors. In contrast, such an association was not observed in malignant midgut endocrine tumors [68].

Table 2
Comparison of 11q Allelic Deletions (LOH) in Pancreatic (Foregut, A) and Mid-/Hindgut (B) Endocrine Tumors, Showing the Specifity of High LOH Frequency with Consistent Extension to the Most Telomeric Marker in the Pancreatic Neoplasms

A

	INSULINOMAS						OTHERS*				MEN-1				NON FUNCTIONING										
Case #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
B/M	*B*	*B*	*B*	*B*	*B*	*B*	*B*	*B*	*M*	*M*	*M*	*M*	*B*	*B*	*B*	*B*	*B*	*B*	*B*	*B*	*M*	*M*	*M*	*M*	*M*	*M*	*M*
PYGM	●	○	○	★	○	○	●	○	○	○	●	●	●	●	○	○	○	●	●	●	○	●	●	●	●	●	●
D11S4946	●	○	○	●	★	★	●	★	★	○	●	●	★	●	○	○	○	★	●	★	★	★	●	★	★	★	●
D11S913	★	★	★	●	○	★	●	○	○	○	●	★	★	●	○	○	★	●	●	★	○	●	●	★	★	●	●
D11S916	★	○	○	●	○	○	●	○	○	○	●	●	●	●	○	★	○	★	★	●	★	★	●	●	●	●	●
D11S901	●	○	○	●	○	○	●	○	○	○	●	●	●	●	○	○	○	★	●	★	○	●	●	●	●	●	●
D11S1365	★	○	★	●	★	★	★	○	○	○	●	★	●	★	○	○	○	●	●	●	★	●	●	★	●	●	●
D11S29	●	★	○	●	-	-	●	-	-	-	●	●	●	●	-	○	★	-	●	●	○	●	●	●	●	●	●
D11S387	●	○	○	●	-	-	●	-	-	-	●	●	●	★	-	○	○	-	★	●	○	●	●	●	●	●	-

B

	ILEAL																APPENDICEAL							RECTAL		
Case #	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53
B/M	*M*	*M*	*M*	*M*	*M*	*M*	*M*	*M*	*M*	*M*	*M*	*M*	*M*	*M*	*M*	*M*	*M*	*B*	*B*	*B*	*B*	*B*	*B*	*B*	*M*	*B*
PYGM	○	○	-	★	○	○	○	○	○	○	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○
D11S4946	★	★	-	○	★	★	★	★	○	○	○	●	○	○	○	○	○	○	○	○	○	★	★	○	★	○
D11S913	★	-	-	○	★	○	★	○	★	★	○	●	○	○	○	○	★	○	○	○	★	○	○	○	○	★
D11S916	○	○	○	○	○	●	★	○	○	○	○	○	○	○	○	○	★	○	○	○	○	○	○	○	○	★
D11S901	○	○	○	○	○	●	○	○	○	★	○	●	★	○	○	○	★	○	○	○	○	★	○	○	○	○
D11S1365	○	○	○	★	○	●	★	★	○	○	○	○	○	○	★	○	○	★	○	○	○	○	○	○	★	○
D11S29	-	-	-	○	○	-	○	●	○	○	★	●	○	-	-	-	○	-	-	○	○	○	○	-	-	-
D11S387	-	-	-	●	○	○	●	○	○	○	○	●	○	-	-	-	○	-	-	○	○	○	○	-	-	-

Modified from ref. 63.

Chromosomal location of microsatellite markers: *PYGM, D11S4946, D11S913*: 11q13; *D11S916*: 11q13–q23; *D11S901, D11S1365*: 11q14; *D11S29*: 11q23; *D11S387*: 11q25;

*Includes heterogeneous noninsulinomas functioning tumors;

B, Benign; M, malignant; ●, loss of heterozygosity; ○, retention of heterozygosity; ★, not informative; –, not done or not evaluable.

Hypermethylation at the 5' region of the $p16^{INK4a}$ tumor suppressor gene on chromosome 9p was observed in the absence of homozygous deletion or mutation in duodenal G-cell tumors [70]. Similar findings were obtained in four of nine midgut EC cell tumors [71].

The imbalance of chromosome 18, with frequent loss of 18q, is a frequent abnormality of well-differentiated gastrointestinal endocrine tumors and appears to be typical of midgut "carcinoids" (EC cell tumors), as described by both comparative genomic hybridization (CGH) and LOH studies [72–76]. In addition, LOH for markers of the deleted in colorectal carcinoma (*DCC*) gene on chromosome 18q21 was reported in well-differentiated and poorly differentiated carcinomas of the stomach [77].

No *p53* gene mutation was detected in well-differentiated gastric, small intestinal, and appendiceal "carcinoid," while one out of nine tumors of the colon–rectum proved mutated [78]. Indeed *p53* gene abnormalities and hyperexpression–accumulation are restricted mostly to poorly differentiated endocrine carcinomas of the gut [77,79,80].

In conclusion, it seems clear that consistent and firm data support a pathogenetic role for the *MEN1* gene only for tumors of the upper gut. Similar evidence is, however, missing for midgut and lower gut tumors. In addition, as a substantial fraction of sporadic tumors at any gut location still lacks any *MEN1* gene involvement, other transformation pathways have to be hypothesized and explored. The present evidence may suggest a possible transformation pathway involving yet unknown oncosuppressor gene(s) in 18q, especially in midgut tumors. *p53* gene involvement is consistently restricted to aggressive poorly differentiated endocrine carcinomas of the gut.

DIAGNOSIS

In agreement with the new formulation of the WHO classification [81], gut endocrine tumors are classified according to anatomical location, tumor cell type, and differentiation status of tumor cells. A uniform scheme of classification is used for all sites and is based on three main categories, one of which is subdivided further into two subgroups:

1. Well-differentiated endocrine tumors, including tumors with benign behavior (1.1) and tumors with indefinite behavior (1.2) at diagnosis.
2. Well-differentiated endocrine carcinomas, low grade.
3. Poorly differentiated endocrine carcinomas, high grade.

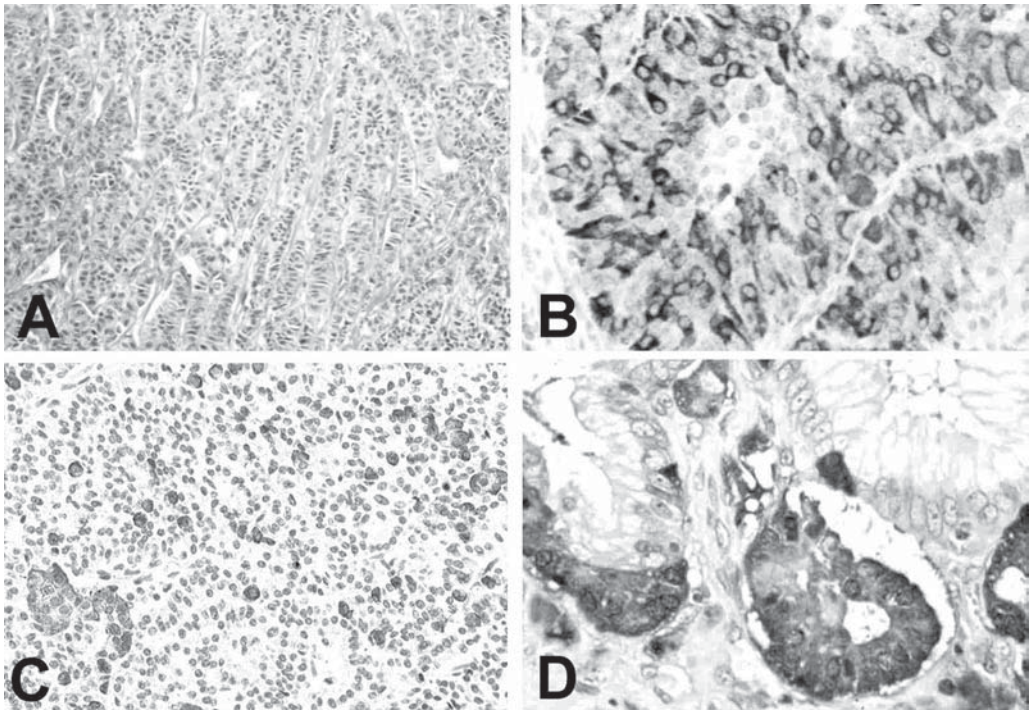

Figure 3 Features of well differentiated endocrine tumors of the gut. (**A**) Low-power micrograph illustrating the mostly trabecular structure of a cecal tumor; note the regular and monomorph tumor morphology. (**B**) Diffuse although variably intense chromogranin A expression in a lymph node metastasis from a sporadic (type III) gastric tumor. (**C**) Ghrelin-expressing tumor cells either isolated (*center*) or in clusters (*lower left corner*) in a gastric endocrine tumor associated with hypergastrinemia and atrophic gastritis (type I); note the regularly packed trabecular structure. (**D**) Somatostatin-expressing cells in a duodenal D cell tumor; note the isolated normal somatostatin cell in the mucosa (*upper right corner*) and the typical glandular structure (*center*). Hematoxylin & eosin (**A**); immunoperoxidase, ABC method; hematoxylin counterstain (**B–D**).

The latter group comprises highly malignant tumors virtually never associated with a hormone-related clinical syndrome displaying an undifferentiated, although protoendocrine, phenotype. The life expectancy of patients with poorly differentiated endocrine carcinomas is comparable to, if not poorer than, that of patients with undifferentiated exocrine carcinomas and thus requires aggressive treatment. Because of the severe clinical implication of such diagnosis, discriminating between well-differentiated and poorly differentiated endocrine tumors is of paramount importance. In the following paragraphs we provide a simple guide for the assessment of parameters useful for the identification of these two tumor categories and for further refining the well-differentiated tumor definition.

Independently from tumor site, a practical diagnostic algorithm for routine diagnostic pathology includes (1) the identification of the typical histology of such tumors; (2) the assessment of tumor cell differentiation status; (3) the identification of the prevalent endocrine cell type, when applicable; and (4) the assessment of predictors of malignancy.

HISTOLOGY In general, well-differentiated tumors are characterized by bland features; trabecular, glandular, acinar, or mixed structures [82]; and tumor cell monomorphism with abundant variably eosinophilic cytoplasm, low cytological atypia, and low mitotic index (Fig. 3). On the contrary, poorly differentiated carcinomas usually display prevalent solid structure with abundant necrosis, often central, round tumor cell of small to medium size with severe cellular atypia, and high mitotic index (Fig. 4) [81].

DIFFERENTIATION STATUS This is assessed by immunohistochemistry for general and specific endocrine markers [19]. In pathology practice widely used general markers are either the cytosol markers NSE and PGP 9.5, or the vesicular markers chromogranin A and related fragments (associated with large, dense-core vesicles) and synaptophysin (associated with small synaptic-like vesicles) (see under subheading Histogenesis for references). In the routine assessment of endocrine differentiation, it is expected that well-differentiated tumor cells express diffusely and intensely most if not all the above-mentioned markers as the normal endocrine cell counterparts (Fig. 3B,C).

In contrast, poorly differentiated carcinoma cells are in general negative for chromogranin A, although diffusely positive for NSE, PGP 9.5, and synaptophysin (Fig. 4B,C). These features reflect the rarity of large dense-core, electron-dense granules observed in poorly differentiated carcinomas cells by electron microscopy [83]. The absence of chromogranin A and of hormone gene expression (see below) in poorly differentiated endocrine carcinoma cells is consistent with the demonstrated "on/off" switch function exerted by the chromogranin A gene in mammalian cells [84]. In routine practice, a diffuse and strong positive stain for at least two of the above-mentioned markers is required for the diagnosis of poorly differentiated

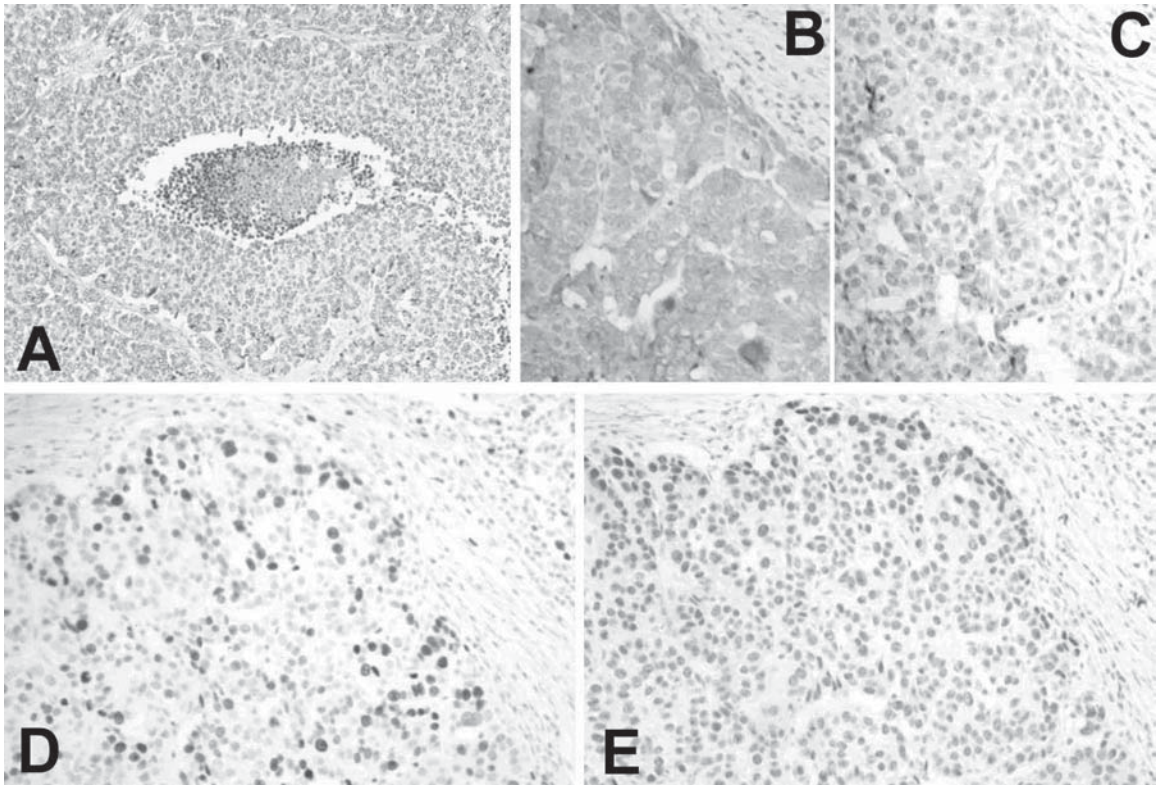

Figure 4 Features of poorly differentiated endocrine tumors of the gut. (**A**) Solid structure with central necrosis of a poorly differentiated endocrine carcinoma of the stomach; the severely atypical tumor cells are tightly packed. (**B,C**) Intense and diffuse synaptophysin immunoreactivity (**B**) and faint, focal chromogranin A expression (**C**, *upper left corner*) in a poorly differentiated endocrine carcinoma of the colon. (**D,E**) Intense and diffuse nuclear immunostaining for Ki-67 (**D**) and *p53* (**E**). Note that a significant fraction of tumor cells are Ki-67 positive while almost all of them display accumulation/hyperexpression of *p53*; same case shown in **B** and **C**. Hematoxylin and eosin (**A**); immunoperoxidase, ABC method; hematoxylin counterstain (**B–E**). (Panels D and E reproduced with permission from ref. ***90***.)

endocrine carcinoma. High Ki-67 index (Fig. 4D) and extensive *p53* hyperexpression/accumulation (Fig. 4E) are additional features typical of the highly malignant poorly differentiated endocrine carcinoma (see the following paragraph).

TUMOR CELL TYPING In well-differentiated endocrine tumors hormone-specific sera (specific markers) readily identify specific tumor cell types (Fig. 3D). This assessment allows the tumor cell type subclassification either in presence or in absence of hyperfunctional syndrome. On the contrary, no hormone immunoreactivity is usually detected in poorly differentiated carcinomas.

PREDICTORS OF MALIGNANCY The clinical behavior of gut endocrine tumors spans from benign to low-grade malignant for well-differentiated tumors/carcinomas, to highly malignant for poorly differentiated carcinomas. The definition of well-differentiated carcinoma is restricted to well-differentiated endocrine tumors with proven malignancy at diagnosis, that is, presenting synchronous metastasis and/or deep wall invasion [81]. Notably, with the exception of insulinomas, hyperfunctional activity bears unfavorable prognostic significance and is consistently associated with either potential or overt malignancy.

However, in the absence of such evidence the behavior of well-differentiated tumors may be unpredictable so that such tumors are either classified as benign or of uncertain behavior according to the presence of given variables. The variables and relative cut-offs are different for tumors arising in different anatomical locations. (It is suggested that the reader refer to the WHO blue book for reference [81].) Finally, the malignant potential is not related to tumor cell type.

A number of clinicopathological parameters, some of which are included in the new WHO classifications, may be of help in assessing the malignant potential in the absence of proven malignancy. Such parameters include tumor size (larger tumors usually are more aggressive); invasion of nearby tissue (pancreas or appendix) or wall invasion beyond the submucosa; angioinvasion and invasion of perineural spaces; solid, nonorganoid structure; necrosis; overt cell atypia; ploidy status (aneuploidy correlates with poor prognosis) [85]; more than two mitoses in 10 microscopic fields at high power (HPF); Ki-67 index of more than 2% or of more than 100 in 10 HPF (Fig. 4D); loss of chromogranin A immunoreactivity, argyrophilia, or hormone expression and nuclear *p53* protein hyperexpression/accumulation (Fig. 4E).

The predictive value of some of these variables was demonstrated in retrospective studies of large series of pancreatic and gastric tumors [80,85–87]. Specifically, tumor size, angioinvasion, mitotic index, and Ki-67 emerged as independent predictors of malignancy and survival in gastric endocrine tumors

[80]. However, mid- and lower-gut endocrine tumors have not been systematically investigated for most of these variables and, for some of them, the published data are scant and contradictory. In small series of midgut well-differentiated tumors, the Ki-67 index was initially suggested as a potential marker of malignancy [88,89]. However, in a subsequent study we failed to confirm this indication showing Ki-67 values similar to those of benign digestive endocrine tumors [90]. In rectal carcinoids a single study showed a very low Ki-67 index (median, 0.62%) [91]. Although unrelated to clinicopathologic features such as infiltration of the muscularis propria, lympho- or angioinvasion, or lymph node metastases, the Ki-67 index was significantly higher in tumors >0.5 cm in size and positively correlated with transforming growth factor-α (TGF-α) expression [91]. Finally, in small series of midgut carcinoids, the ploidy status appear not to correlate with malignancy [92,93], as also confirmed in a recent investigation [68]. In contrast, aneuploid status was found in three malignant rectal carcinoids [94].

The major limit of these data is the small size of the tumor series investigated. Extensive work on large and homogeneous tumor series is therefore required to characterize better malignancy-associated parameters for intestinal endocrine tumors.

DIFFERENTIAL DIAGNOSIS

WELL DIFFERENTIATED ENDOCRINE TUMOR Independently from the different tumor types observed at different anatomical locations, the differential diagnosis usually occurs between deeply invasive well-differentiated endocrine carcinoma and the conventional adenocarcinoma when displaying solid/trabecular structure. The presence of both positive mucin stain (periodic acid-Schiff [PAS]/Alcian blue or PAS after diastase digestion) and negative immunohistochemistry for endocrine markers supports the diagnosis of conventional adenocarcinoma. In contrast, the presence of rare carcinoma cells positive for endocrine markers (e.g., silver impregnation or chromogranin A) is a frequent finding [95,96], although irrelevant for the diagnosis of adenocarcinoma as well as for the clinical outcome of the patient [97]. However, if the endocrine tumor cell fraction is balanced with the adenocarcinoma cell fraction, a diagnosis of mixed exocrine–endocrine carcinoma is indicated.

POORLY DIFFERENTIATED ENDOCRINE CARCINOMA The most frequent differential diagnosis occur between poorly differentiated endocrine carcinoma and nonendocrine adenocarcinoma, poorly differentiated, mostly of solid type. Although the solid structure with central necrosis is of help in suspecting a generic "endocrine" carcinoma diagnosis, these features are frequently underestimated as the severe cyto/histological atypia and the confounding, often diffuse, necrosis and inflammatory infiltrate. The presence of positive mucin stain in more differentiated areas and the absence of positive immunohistochemistry for general markers of endocrine differentiation (i.e., synaptophysin, NSE, or PGP9.5) in the majority of carcinoma cells support the diagnosis of adenocarcinoma, poorly differentiated.

WELL-DIFFERENTIATED TUMOR TYPES

STOMACH Different cell types, namely histamine ECL, somatostatin D, serotonin EC, ghrelin P/D_1, and gastrin G cells, may compose gastric well-differentiated endocrine tumors. However, ECL cell tumors are by far the most frequent finding.

ECL Cell Tumors Commonly defined as gastric carcinoids, these are composed of histamine-producing ECL cells of the oxyntic mucosa and produce histamine and the molecules involved in its synthesis and intracellular processing such as histidine decarboxylase and VMAT2. In addition, rare tumor cells may express other hormones such as serotonin, gastrin, and ghrelin [83,98,99].

Three independent types of ECL cell tumors have been identified on the basis of the associated pathological conditions [83].

Type I tumors, associated with atrophic corporal gastritis (ACG), account for 70–80% of all gastric (ECL cell) carcinoids. ACG and the related achlorhydria is the causative condition for secondary hypergastrinemia of antral origin that is consistently associated with these tumors and regarded as the initiating trophic stimulus for ECL cell proliferation [100]. Type I tumors more frequently affect females (75% of cases). More frequently multiple, type I tumors usually appear as small, clinically silent polyps generally limited to the mucosa or the submucosa. At histology, they display a typical carcinoid structure and are consistently associated with hyperplastic/dysplastic proliferation of extratumoral ECL cells. Metastases to regional lymph nodes are rare (fewer than 5%) and to distant sites exceptional. No cases of tumor-related death are on record [101]. From the clinical point of view type I ECL cell carcinoids are consistently nonfunctioning.

Type II tumors are associated with MEN1, usually with Zollinger–Ellison syndrome (ZES), and account for approx 6% of ECL cell carcinoids. Usually multiple, type II tumors are larger than those of type I, although overall <1.5 cm in size in 73% of cases [101]. Histologically, type II tumors are similar to type I with the associated feature of precursor lesions of ECL cells in extratumoral mucosa. Metastases to lymph node are present in 30% of patients [101]. The prognosis of these tumors is usually good but cases with very aggressive course have been described [55]. In general, however, the patient's prognosis depends more on the background MEN 1 setting. From the clinical point of view, type II ECL cell tumors are nonfunctioning, the associated ZES being caused by the concomitant gastrinomas of the pancreas or, more often, of the duodenum.

Type III or sporadic ECL cell tumors are not associated with hypergastrinemia, extratumoral proliferation of ECL cells, or other significant pathological conditions of the stomach; account for 14–25% of all ECL cell carcinoids; and show a striking male predominance (74%) [101]. Type III tumors are usually single and in 33% of cases >2 cm in diameter. Infiltration of the muscularis propria is observed in 76% of cases and of the serosa in 53%. Although most type III tumors are histologically typical carcinoids, more atypical features may be found especially in neoplasms exceeding 2 cm in size [80,102]. Metastases occur in three fourths of patients, especially in tumors with atypical features, and are often located to the liver. Tumor-related death is seen in 27% of cases with a median survival of 28 mo [101]. From the functional point of view, type III ECL cell carcinoids are the only type of ECL cell tumor that may be associated with a clinical syndrome. This is a variant of the classical carcinoid syndrome showing a cherry red rather than cya-

notic flushing but no diarrhea, bronchoconstriction, or heart disease. The syndrome is often referred to as "atypical (or foregut) carcinoid syndrome"; includes facial edema, rhinorrhea, salivation, and lacrimation; and is considered to depend on tumor release of 5-hydroxytryptophan and/or histamine [103].

SMALL INTESTINE–DUODENUM Although somatostatin D, serotonin EC, and gastrin G cells may compose duodenal well-differentiated endocrine tumors, G- and D-cell tumors are those more frequently reported.

G-Cell Tumors Accounting for about two thirds of all duodenal neuroendocrine tumors, duodenal G-cell tumors are preferentially located in the proximal duodenum and are usually small (<1 cm in diameter) [104]. Metastases are common, even in the smallest tumors, usually confined to regional lymph node(s). Liver metastases are rare and, usually, late events. Duodenal G-cell tumors may be either sporadic or associated to MEN 1 syndrome roughly in a ratio of 2:1. As a rule MEN 1 tumors are multicentric and may escape detection even at surgery [105]. Both sporadic and MEN 1 associated duodenal G-cell tumors are often but not always associated with ZES; in the latter case a definition of gastrinomas is accepted.

D-Cell Tumors These tumors occur with a frequency similar to that of pancreatic D-cell tumors but tend to be smaller [106]. They are malignant in the same rate (50%) but metastases to regional lymphnodes are much more frequent than those to the liver. At histology, D-cell tumors are characterized by a glandular pattern with frequent calcified psammoma bodies. At variance with their pancreatic counterpart they are often associated with type 1 neurofibromatosis and are not associated with the functional syndrome.

SMALL INTESTINE–ILEUM AND CECUM Although at least 10 different endocrine cell types are reported in this part of the gut, only serotonin EC, rare somatostatin D (*see* above), and enteroglucagon L-cell tumors (*see* below) are usually reported.

EC-Cell Ileal Tumors Accounting for the vast majority of endocrine tumors of the ileum and cecum, EC-cell tumors are characterized by expression of serotonin and substance P. Usually >1 cm in size at diagnosis, EC-cell tumors are multiple in up to 40% of cases and tend to infiltrate deeply the muscular wall with frequent metastases to lymph node [104]. Their histological structure is typical, showing solid clusters arranged in an insular pattern. Proliferation of EC cells in contiguous crypts may suggest possible precursor changes [107]. Owing to the release of serotonin and other active substances, an overt carcinoid syndrome is present in about 20% of cases and strictly depends on the establishment of liver metastases. It presents with the typical combination of flushing and diarrhea, sometimes associated with bronchoconstriction-dependent asthma and right heart disease [108].

EC-Cell Tumors of the Appendix Accounting for the overwhelming majority of appendicular endocrine tumors, EC-cell tumors exhibit the same histological appearance and immunohistochemical features of their midgut counterpart. In contrast, appendix EC-cell tumors appear to originate from the submucosal neuroendocrine complexes closely associated with Schwannlike, S100 immunoreactive, sustentacular cells [104, 107]. Appendicular EC cell tumors tend to be small, discovered serendipituously, in the absence of the "carcinoid" syndrome and with almost invariably benign course even if infiltration of the muscularis propria is common. Only tumors >2 cm in size or infiltrating the mesoappendix are a potential source of metastasis.

LARGE INTESTINE Similar to what was reported for the small intestine, in spite of the various normal cell types present in the large intestine only L-cell and, more rarely, EC-cell tumors (*see* earlier) are reported.

Rectal L-Cell Tumors These tumors are usually composed of L cells producing peptides of the glucagon–glicentin and PP–PYY families [109,110]. In this respect, rectal carcinoids are fully different from those of the remaining large bowel mostly composed of serotonin-producing EC cells. Rectal L-cell carcinoids are in general small, often polypoid neoplasms, expanding in the submucosa and showing a distinctive trabecular structure. From the clinicofunctional point of view they are usually silent. Their clinical behavior is mostly benign but tumors >2 cm in diameter or infiltrating the muscularis propria may metastatize and pursue an unfavorable course.

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16 The Endocrine Pancreas

STEFANO LA ROSA, MD, DANIELA FURLAN, BSCD, FAUSTO SESSA, MD, AND CARLO CAPELLA, MD

THE NORMAL ENDOCRINE PANCREAS

HISTORICAL BACKGROUND The endocrine function of the pancreas was first proposed in 1889 by Von Mering and Minkowsky [1]. A few years later, Laguesse [2] attributed this function to the islets of Langerhans [3]. Diamare identified two islet cell types [4] and, successively, Lane [5] and Bensley [6] named these cells α and β. Just a few years later, other investigators described δ and the fourth type (PP) of islet cells [7,8]. The use of immunohistochemical methods helped in the understanding of the functions and roles of islet cells, correlating the morphological features with the hormonal products. Thus it was demonstrated that β cells produce insulin [9], α cells glucagon [10], δ cells somatostatin [11], and PP cells (F and D_1 cells) pancreatic polypeptide [12,13]. Following these pioneer studies, further investigations using new technical approaches, including electron microscopy, *in situ* hybridization, and molecular biology, have better elucidated the biological role and functions of pancreatic hormones, giving more detailed information about the physiopathology of pancreatic endocrine diseases such as diabetes mellitus and pancreatic endocrine tumors.

MOLECULAR ASPECTS OF ENDOCRINE PANCREAS DEVELOPMENT In the human fetus the primitive pancreas appears during the fifth week of gestation as two evaginations, one dorsal and one ventral, from the primitive foregut [14]. During the sixth week the primitive intestine and its derivates turn 90° clockwise, and at the end of this rotation the ventral pouch adheres to the dorsal one [14]. The endocrine cells appear during the eighth week as single elements scattered at the base of the primary tubules. The α, β, and δ cells appear at about the same period while PP cells follow a few days later [14]. In addition, recent studies in mice have demonstrated an expression of peptide tyrosine–tyrosine (PYY) [15] and a transient expression of other gut hormones, such as secretin and gastrin, in early precursors of endocrine cells [16,17], suggesting a peculiar functional plasticity of the developing endocrine pancreas. However, clusters of epithelial endocrine cells forming the primitive islets bud off from the tubules at 10–13 wk [18] and this process seems to occur when endocrine cells start to express certain cell adhesion molecules, such as neural cell adhesion molecule (N-CAM) and cadherins [19,20].

Transcription Factors in Endocrine Pancreas Differentiation In recent years several attempts have been made to understand the mechanisms underlying pancreatic islet development and differentiation. Current knowledge about endocrine cell lineage of the pancreas originates from ablation studies [21–23], analyses of coexpression of different hormones [24–26], transgenic approaches [15,27], and molecular biology investigations [28–34]. The most important contributions to the field derive from the study of the phenotypes of mouse pancreatic cell lines with induced mutations of developmental control genes, including *HNF-1α* [35], *Isl-1* [36], *beta2/NeuroD* [37], *Nkx2.2* [38], *p48* [39], *Pax4* [40], *Pax6* [41,42], *IPF-1/pdx1* [43,44], and *neurogenin 3* [45]. All these genes encode for specific transcription factors that belong to two major classes of regulatory proteins called homeodomain factors (HDs) and basic helix–loop–helix (bHLH) factors [46].

Neurogenin 3 (*ngn3*), a member of the bHLH transcription factor family, is involved in the determination of neural precursor cells in the neuroectoderm. *Ngn3* is expressed in different regions of the nervous system and in scattered cells of the embryonic pancreas [45]. Gradwohl et al. [47] demonstrated that mouse embryonic *ngn3*-positive cells coexpress neither insulin nor glucagon, suggesting that *ngn3* marks early precursors of pancreatic endocrine cells. Mice lacking *ngn3* function fail to generate any pancreatic cells and die of diabetes postnatally. Expression of *Isl-1, Pax4, Pax6*, and *NeuroD* is lost and endocrine precursors are lacking in the mutant pancreatic epithelium. Thus, *ngn3* seems to be mandatory for the specification of a common precursor for the four pancreatic endocrine cell types. Interestingly, proteins of the bHLH family are important transcriptional regulators of neural differentiation in both invertebrate and vertebrate species. For example, the proneural bHLH genes *achaete-scute* and *atonal* are required for the determination of neural precursors in *Drosophila*. Similarly, the mouse neurogenin 1 (*ngn1*) and neurogenin 2 (*ngn2*) are essential for the determination of sensory lineages in the peripheral nervous system [48,49]. In initiating neuronal differentiation, bHLH factors use the Notch-signaling pathway [50,51], a molecular machinery that is also involved in the picking out of

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Table 1
Main Transcription Factors Involved in Pancreatic Development

Transcription factor	*Family*	*Expression in adult pancreas*	*Null mutant pancreatic phenotype*
IPF-1/Pdx1	HD	β cells	Pancreas agenesis
Isl-1	HD	α, β, δ, PP cells	No islet cells
Nkx6.1	HD	β cells	Not known
Nkx2.2	HD	α, β, PP cells	β cell absence α, PP cells reduction
Pax4	HD	β-, δ, PP cells	β, δ cells absence Increase in α cells
Pax6	HD	α, β, δ, PP cells	α cells absence
Hlxb9	HD	β cells	Dorsal bud agenesis, perturbed ventral endocrine differentiation
Neurogenin 3	bHLH	Endocrine precursor cells	
NeuroD	bHLH	α, β, δ, PP cells	Endocrine cell reduction
p48	bHLH	Exocrine cells	No exocrine pancreas, islet cells in the spleen

HD, Homeodomain factors; bHLH, basic helix–loop–helix factors.

Table 2
Growth Factors and Growth Factor Receptors Expression in Adult Pancreatic Islet Cells

	Expression in adult islet cells	*References*
Growth factors		
FGF7	A few islet cells	[83]
HGF	α, β cells	[434]
Activin A	α cells	[95]
Activin B	β cells	[96]
TGF-α	All types	[432]
TGF-β_{1-3}	β cells	[104]
VEGF	β, PP cells	[112,113]
Growth factor receptors		
FGFR1	α cells	[84]
FGFR2	β cells	[82–84]
FGFR3	Rare	[84]
FGFR4	α, β, PP cells	[84]
met	β cells	[434]
VEGFR1	Rare	[113]
Follistatin	β cells	[94]

the scattered subset of endodermal cells destined for an insular endocrine fate [52].

The homeodomain transcription factor *IPF-1/pdx1* (insulin promoter factor 1, also known as the murine homologue *pdx1*, pancreatic duodenal homeobox) seems to be a critical regulator of pancreatic development in humans [53] as well as in mice [43]. *IPF-1/pdx1* gene expression provides specific functions both in the regionalization of the primitive gut endoderm and in the maturation of the pancreatic β cell. The gene is transiently expressed in all pancreatic cells as well as in the epithelial layer of the duodenal mucosa during embryogenesis [54,55]. As development proceeds *IPF-1/pdx1* becomes progressively confined to endocrine β cells, where it plays a critical role in the transactivation of genes associated with β-cell identity, including insulin, glucose transporter 2, glucokinase, and islet amyloid polypeptide [56–58]. The homozygous deletion of the gene in mice [43,59] and in a patient [53] causes pancreatic agenesis. On the contrary, *IPF-1/pdx1* heterozygous mutations are associated with type 2 adult onset diabetes [44,60].

Some homeodomain transcription factors such as *IPF-1/pdx1, Hlxb9, Pax4,* and *NKx6.1* display an expression restricted to β cells [33,40,55,61–64], whereas others such as *Pax6* [65], *Isl-1* [66], and *Nkx2.2* [38] are expressed in more than one islet cell type. Within the well-documented *PAX* (paired box) family of transcription factors involved in the embryogenesis of many organs, *Pax4* and *Pax6* seem to encode key regulators of the differentiation of specific endocrine cell lineages during pancreas development [67]. Mice homozygous for a null mutation in the *Pax4* gene have a marked decrease in β and δ cells and an increase in α cells [40]. In contrast, *Pax6* is specifically involved in the differentiation of α cells [41]. Furthermore, double null mutants for both *Pax4* and *Pax6* fail to produce any mature pancreatic endocrine cells. It has been suggested that these two factors together are required for endocrine cell differentiation and that *Pax4* may function as a transcriptional repressor competing with *Pax6* for the same promoter binding site [41]. *Nkx2.2* is expressed in α, β, and PP cells but *Nkx2.2* mutants lack only β cells. In addition, *Nkx2.2* is required for maintaining *Nkx6.1* expression in mature β cells [38].

Taken together these data demonstrate that transcription factors belonging to either HD or bHLH classes (Table 1) seem to play a central role in pancreatic endocrine cell ontogeny as well as in endocrine cell differentiation and development [68] and that the initial development of pancreatic endocrine cells relies on molecular mechanisms similar to those involved in neuronal development, although pancreatic endocrine cells derive from endodermal progenitor cells.

Growth Factors in Endocrine Pancreas Differentiation

Growth factors are diffusible molecules that play a role in endocrine pancreas differentiation [69,70]. They act by controlling the proliferation of immature epithelial cells, stimulating their differentiation into endocrine or exocrine cells and favoring the aggregation of endocrine cells to form the islets of Langerhans. Moreover, the different distribution of growth factors in the different types of normal endocrine pancreatic cells of adults (Table 2) suggests that they may also play a biological role in regulating the cell functions of the islets.

Recent studies [71–79] have shown that different fibroblast growth factors (FGFs) and their receptors (FGFRs) are expressed during pancreatic development and that islet development is disturbed when the FGF–FGFR interactions are perturbed [80]. Mice transgenic for the hybrid apolipoprotein E (*Apo E*)/*FGF7* gene develop an increased number of periductal islets and show a marked increase in insulin expression in ductule epithelial cells [81]. In addition, when the signals mediated by FGFR2 (the receptor specific for *FGF7*) are blocked in mice rendered trans-

genic for the dominant negative soluble receptor, no islets are found in the pancreas [82], suggesting that signals trasduced via FGFR2 are important for pancreatic development. In this context, it is interesting to note that both FGF7 and FGFR2 [83,84] have been detected in the islets of human adults.

Members of the transforming growth factor-β (TGF-β) superfamily also seem to be involved in endocrine pancreas development. The TGF-β superfamily includes several soluble factors, such as activins, inhibins, and various TGF-β isoforms [85,86]. Activins induce the expression of pancreatic genes during early development [87] and in cultures from chick embryos [88]. In addition, activins, in combination with either beta cellulin or hepatocyte growth factor (HGF), are able to convert AR42J cells, representing a sort of pluripotent precursor cells, into insulin-secreting cells [89,90]. At least in fetal rat pancreas, activin A has a different cell expression during embryogenesis and its modulatory role in the development of islet cells has been suggested [91]. Various types of activins and inhibins have also been demonstrated in adult rat and human islet cells [92–96], suggesting their possible role in the regulation of the adult endocrine pancreas, including the ability of activin A to stimulate insulin secretion by pancreatic islet cells [97]. A main role of activin in islet cell growth and differentiation is also indirectly suggested by mice rendered transgenic for the activin receptor mutants, under the control of the insulin or β-actin promoter, in which hypoplasic pancreatic islets are detected [98,99]. The actions of activins are in part modulated by follistatin, a protein not belonging to the TGF-β superfamily [86]. Follistatin itself is known to regulate the relative proportion of endocrine vs exocrine pancreatic tissues during development by promoting the development of amylase-expressing cells from immature pancreatic epithelial cells and repressing the development of insulin cells [100]. Interestingly, follistatin has been localized in human adult pancreatic islet cells [94,101].

Different experimental approaches have been used to establish whether TGF-β isoforms could be implicated in pancreatic development. TGF-β_{1-3} isoforms are expressed during pancreas development [102,103] as well as in the adult pancreas [104]. In E12.5 mouse embryo exogenous TGF-β_1 induces a developmental block of acinar cells and, on the contrary, the stimulation of endocrine cell development, suggesting a role of TGF-β_1 in the regulation of the balance between the acinar and endocrine component of the pancreas [103].

The role of transforming growth factor-α (TGF-α) in pancreatic endocrine cell differentiation is less clear. Studies on transgenic mice and rats have suggested that TGF-α, either alone or in combination with gastrin, may be involved in endocrine pancreatic cell proliferation and differentiation [105,106]. In general, members of the epidermal growth factor (EGF) family, which also includes TGF-α, are considered to play a role in pancreatic differentiation as suggested by the fact that mice lacking members of the EGF receptor family show abnormal pancreatic development including reduced levels of islet cell markers [107].

The implication of the nerve growth factor (NGF)/NGF-receptor axis in islet development is suggested by an in vitro model demonstrating that islet morphogenesis is significantly retarded when the *Trk-A* gene, which encodes for high-affinity NGF-receptor, is inhibited [108].

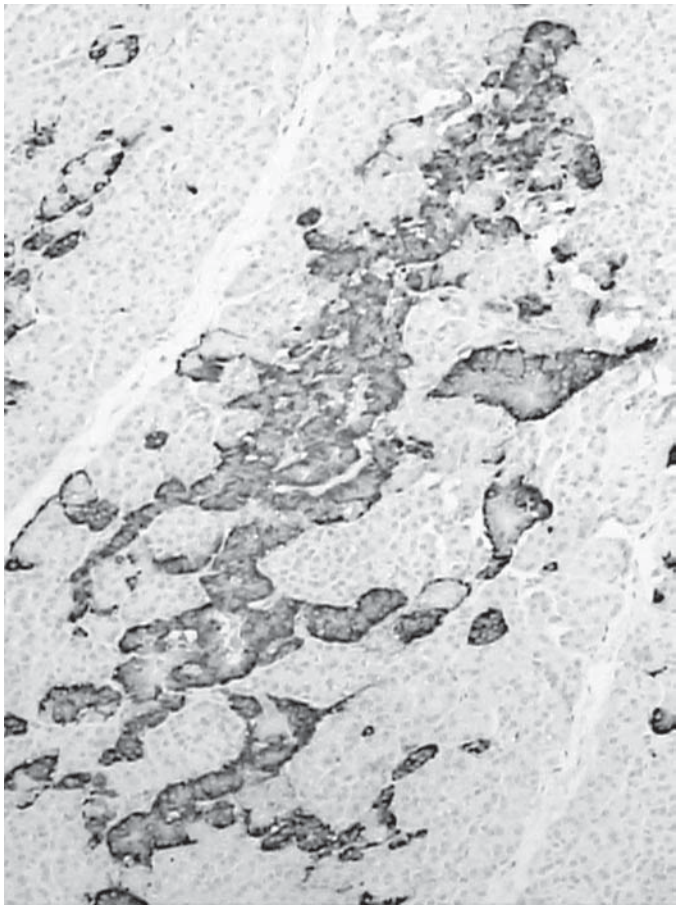

Figure 1 PP immunoreactivity in PP-rich islets of the ventrally derived portion of the pancreatic head.

Vascular endothelial growth factor (VEGF) has no effect on endocrine differentiation of primary cultures of rat duct cells [109] but its flk-1 receptor is needed for β-cell maturation from pancreatic duct cells [110]. In addition, VEGF and its two specific receptors (flt-1 and flk-1) have been identified in islet cells of normal mice [111] and humans [112,113], where they may play a biological role regulating islet cell differentiation and intra-islet capillarization.

MAIN MORPHOLOGICAL FEATURES OF ADULT NORMAL ISLETS Although a small proportion (<10%) of pancreatic endocrine cells are scattered throughout the exocrine parenchyma, most of them are aggregated in the islets of Langerhans. Two types of islets can be recognized in the human pancreas. The islets scattered in the anterior part of the head, in the body, and in the tail of the gland are well demarcated and round to ovoid in shape (regular islets). The islets localized in the posterior part of the head are irregular in shape and are formed mainly by thin trabeculae of perpendicularly oriented cells, most of which are PP secreting (irregular or PP-rich islets) (Fig. 1) [114]. The four main types of islet cells show a different location within the islets (Fig. 2), different ultrastructural features (Fig. 3), and different hormone secretion. The main features of the four islet cell types are summarized in Table 3.

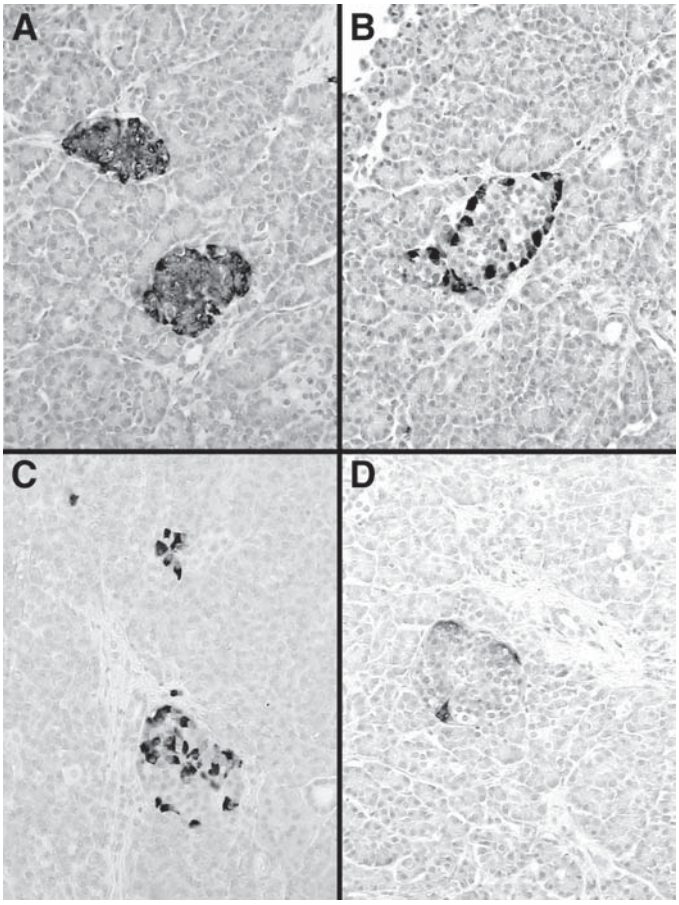

Figure 2 Normal adult human pancreas. Immunoreactivities for insulin (**A**), glucagon (**B**), somatostatin (**C**), and PP (**D**) show the different distribution of β, α, δ, and PP cells in regular islets of the pancreas.

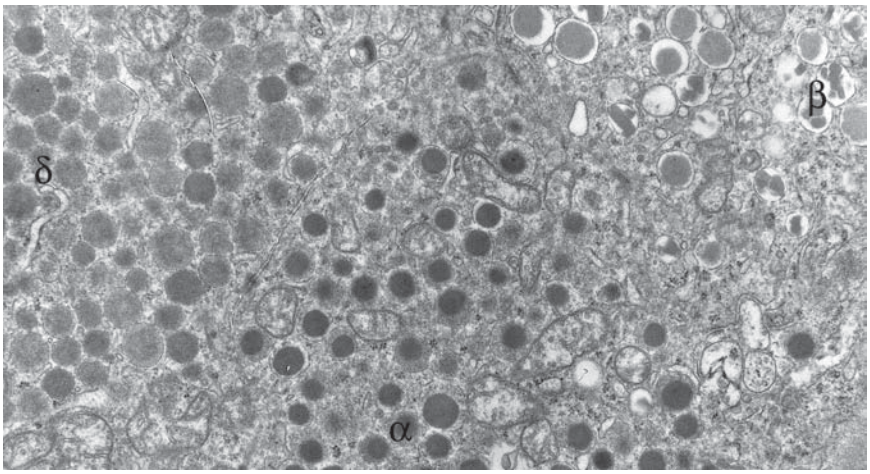

Figure 3 Ultrastructural features of α, β, and δ cells. α cells (**middle**) have secretory granules with centrally or eccentrically located round electron-dense core, which is surrounded by a pale granular halo. β cells (**right**) have secretory granules with both crystalline and noncrystalline core. δ cells (**left**) show large secretory granules with a uniform electron-dense core encircled by a tightly fitting membrane.

α cells constitute 15–20% of the total endocrine mass. They are easily recognized with immunohistochemical techniques using specific C-terminal glucagon sera and appear to be located mainly at the periphery of the islets [115]. In addition to glucagon, PYY peptide as well as general neuroendocrine markers including neuron-specific enolase (NSE), synaptophysin, protein gene product 9.5 (PGP 9.5), chromogranin A and its fragments, and 7B2 have been detected in α cells [116–121]. Ultrastructurally, α cells exhibit characteristic 200–300-nm secretory granules with a central or eccentrically located, round, highly electron-dense core, surrounded by a pale granular halo. The electron-dense core primarily contains glucagon while other pro-

Table 3
Main Features of Human Pancreatic Endocrine Cells

Type	*Hormone*	*Site*	*Granule structure*	*Secretory granule diameter (nm)*
α	Glucagon	Islet periphery	Highly electron-dense core; tightly fitting membrane; gray halo	200–300
β	Insulin	Islet center	Polymorphous core round or paracrystalline; variable electron density; wide clear halo	250–400
δ	Somatostatin	Scattered inside or at the periphery	Large round; moderately electron-dense core; tightly fitting membrane	150–400
PP (D1 typeV)	Pancreatic polypeptide	Periphery of regular islets	Small round; highly electron-dense core; narrow clear halo	90–200
PP (F type)	Pancreatic polypeptide	Bulk of irregular islets	Irregular, angular to round; variable density; tightly fitting membrane	200–300

glucagon-derived peptides (i.e., glucagon-like peptides and glicentin) and chromogranins are stored in the pale halo [122,123].

Insulin-producing β cells represent the prevalent islet cell type of the regular islets and 20–30% of the cells forming the irregular islets [124]. They are easily identified by their immunohistochemical reactivity for insulin, proinsulin, and C-peptide [9,125,126], although amylin (also called islet amyloid polypeptide [IAPP]) is also expressed [127]. Ultrastructurally, β cells contain secretory granules either with a typical crystalloid core or a noncrystalline, finely granular compact core. Crystalline granules contain mainly insulin while compact granules are considered immature and contain proinsulin [128].

δ cells are identified by their immunoreactivity for somatostatin [129]. They are mainly distributed at the periphery of the regular islets and constitute 5–10% of endocrine cells. δ cells typically do not react with Grimelius' silver technique, but stain with alcoholic silver solutions. Ultrastructurally, δ cells show large secretory granules with a moderate and uniform electron density, which are encircled by a tightly fitting membrane [130]. δ cells possess both short processes that contact endocrine cells and long processes that extend to intrainsular capillaries [131]. The distribution of δ cells within the islets and their various cell processes permit the somatostatin secreted by these cells to modulate insulin and glucagon release from β and α cells through paracrine as well as endocrine influences [131].

Although PP cells are argyrophilic with Grimelius' silver stain, the definitive identification is achieved by immunohistochemistry using anti-PP antibodies [12]. In humans, PP cells represent the most frequent (approx 70%) cell type of irregular islets of the posterior part of the head but they account for only 2–5% of endocrine cells in the rest of the pancreas [114], where they are scattered at the periphery of the islets. Ultrastructurally, PP cells occur in two forms: (1) PP cells of the ventrally derived posterior pancreatic head characterized by secretory granules of variable size, shape, density, and inner structure, resembling those of "F cells" of the dog uncinate pancreas; and (2) PP cells of the dorsally derived part showing small secretory granules [13].

In addition to general endocrine markers and specific hormone peptides, islet cells have been found to express several growth factors (Table 2) and myosin XVA (Fig. 4), an unconventional myosin protein, which seems to have a role in secretory granule movement [132].

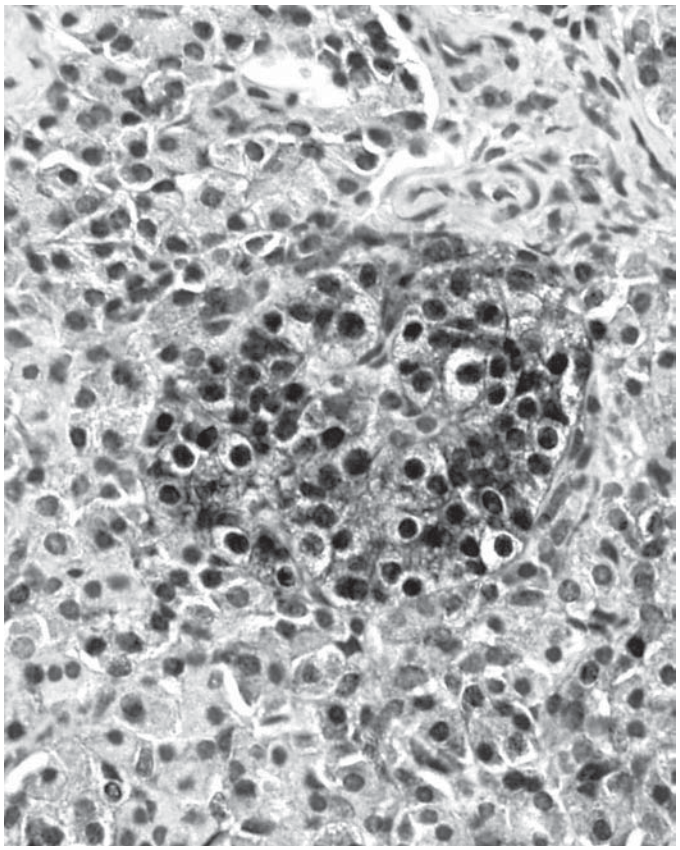

Figure 4 Immunoreactivity for myosin XVA in the majority of cells of a pancreatic islet. (Color illustration appears in insert following p. 148.)

DIABETES MELLITUS

CLASSIFICATION Diabetes mellitus is not a single disease, but rather a heterogeneous group of disorders that share an elevated plasma glucose level due to either an absolute deficiency of insulin secretion or a reduction in its biological effectiveness. The types of diabetes mellitus classified according to the American Diabetes Association and the World Health

Table 4
Etiological Classification of Diabetes Mellitus

I. Type 1 diabetes
  A. Immune-mediated
  B. Idiopathic
II. Type 2 diabetes
III. Other specific types
  A. Genetic defects of β-cell function
    1. HNF-4α (MODY1)
    2. Glucokinase (MODY2)
    3. HNF-1α (MODY3)
    4. IPF-1 (MODY4)
    5. Mitocondrial DNA
    6. Others
  B. Genetic defects in insulin action
    1. Type A insulin resistance
    2. Leprechaunism
    3. Rabson–Mendenhall syndrome
    4. Lipoatrophic diabetes
    5. Others
  C. Diseases of the exocrine pancreas
    1. Pancreatitis
    2. Trauma/pancreatectomy
    3. Neoplasia
    4. Cystic fibrosis
    5. Hemochromatosis
    6. Fibrocalculous pancreatopathy
    7. Others
  D. Endocrinopathies
    1. Cushing syndrome
    2. Acromegaly
    3. Glucagonoma
    4. Pheochromocytoma
    5. Somatostatinoma
    6. Aldosteronoma
    7. Hyperthyroidism
    8. Others
  E. Drug- or chemical-induced
    1. Vacor
    2. Pentamidine
    3. Nicotinic acid
    4. Glucocorticoid
    5. Thyroid hormone
    6. Diazoxide
    7. β-Adrenergic agonists
    8. Thiazides
    9. Clozapine
    10. Protease inhibitors
    11. Others
  F. Infections
    1. Congenital rubella
    2. Cytomegalovirus
    3. Others
  G. Uncommon forms of immune-mediated diabetes
    1. Stiff-man syndrome
    2. Anti-insulin receptor antibodies
    3. Others
  H. Other genetic syndromes sometimes associated with diabetes
    1. Down syndrome
    2. Klinefelter's syndrome
    3. Turner's syndrome
    4. Wolfram's syndrome
    5. Friedreich's ataxia
    6. Huntington's disease
    7. Lawrence–Moon–Biedl syndrome
    8. Monotonic dystrophy
    9. Porphyria
    10. Prader–Willi syndrome
    11. Others
IV. Gestational diabetes mellitus

Modified from ref. [134].

Organization (WHO) [133,134] are reported in Table 4. Type 1 diabetes is due to β-cell destruction which, in more than 95% of cases, is caused by an autoimmune process. Type 2 diabetes, which represents the prevalent form of diabetes, is a disorder characterized by two main metabolic defects: (1) a derangement in β-cell function and (2) a decreased response of peripheral tissues to insulin (insulin resistance). In addition to type 1 and type 2 diabetes mellitus, two other subtypes have been included in the WHO classification: specific types of diabetes (categories for which a cause has been established) and gestational diabetes. There are remarkable epidemiological differences in the incidence and distribution of type 1 and type 2 diabetes [135–137]. The pathogenesis and pathophysiology of diabetes with its clinicopathologic implications is a complex subject that is beyond the scope of this chapter. Thus, the reader is referred to specific texts to obtain more information about this topic [138–140]. In this chapter we will restrict the discussion to the main morphological changes of pancreatic islets in type 1 and type 2 diabetes.

TYPE 1 DIABETES MELLITUS Type 1 diabetes mellitus accounts for approx 10% of all cases of diabetes and occurs most often in young people, often manifesting itself in the form of ketoacidosis that can be treated only with insulin [138]. There are both geographic and race-specific differences in the incidence of type 1 diabetes [135,137].

Morphological changes in the pancreatic gland of type 1 diabetes patients depend on the different stage and duration of the disease. Macroscopically, at the time of clinical onset and for about 1 yr thereafter, the weight and the size of the pancreas are normal [141–143]. However, after 2–5 yr of diabetes, the pancreas becomes smaller and, in some cases, the weight may be <50 g [141,144]. This reduction in pancreatic weight is due to the atrophy of the exocrine parenchyma, which constitutes approx 98% of the pancreatic volume. The atrophy has been attributed to the loss of the high level of insulin that perfuses the acinar tissue through the islet–exocrine vascular connections and that may exert a trophic effect on acinar cells [145,146]. However, the severity of the pancreatic atrophy varies from individual to individual and a relationship between the degree of atrophy and the duration of disease or the age at onset has not been found [144].

The histological features of pancreatic islets are different in the early and in the late phases of the disease. At the time of diagnosis there are pronounced changes in the islets of Langer-

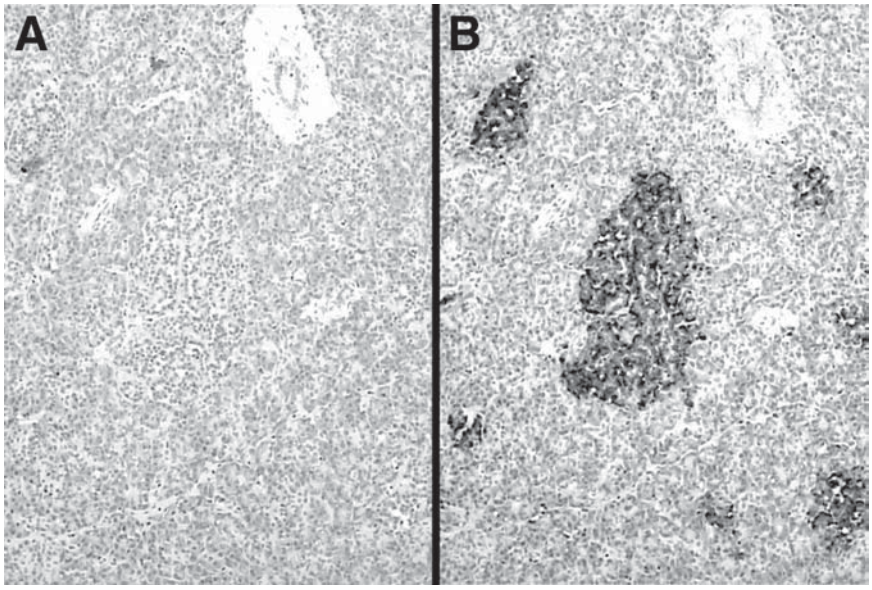

Figure 5 Late phase of type 1 diabetes mellitus. The islets completely lack β cells as shown by negative staining for insulin (**A**), while they are mainly composed of glucagon-positive α cells (**B**).

hans. Three types of islets can be identified [147,148]: (1) The first type are islets showing a marked or total loss of β cells and containing only α, δ, and a few PP cells. The nuclei are small and dark and the cytoplasm is generally scant and eosinophilic. The islets are poorly circumscribed and there is an apparent continuity of the islet cords with the adjacent acini or ducts. (2) The second are oval or round islets that are sharply demarcated from the exocrine parenchyma and tend to be larger than normal. They contain a normal number of β cells that are large, degranulated, and with nuclear hypertrophy, suggesting a functional hyperactivity. (3) The third type are islets with insulitis. Insulitis characteristically appears as an infiltration of some islets (usually not all) by small lymphocytes with scanty cytoplasm. Occasionally, macrophages are present. It should be emphasized that the cellular infiltrate is, with rare exceptions, confined to the islets. The lymphocytes are mainly T cells with only a few B lymphocytes [149–151]. T lymphocytes penetrate the islets from the periphery and destroy β cells by activating apoptotic mechanisms [148]. In recent-onset type 1 diabetes, the insulitis is associated with increased expression of major histocompatibility class (MHC) class I and class II molecules in a minority of β cells [152]. The extent of the insulitis varies from islet to islet and insulitis is generally more prominent in infants, whereas it becomes less evident after the age of 15 yr [149]. In the late (chronic) phase, over a period of years islet degeneration and β-cell loss become severe, although a complete disappearance of islets rarely occurs. Total islet volume is reduced, averaging less than one third the volume of nondiabetic controls and the mean size of the islets is decreased [153]. β cells are virtually absent or consistently reduced. The regular islets are composed mainly of α and δ cells (Fig. 5) while the irregular islets of the dorsal part of the head are composed of PP cells [143,154]. Although islet amyloidosis is normally absent in type 1 diabetic islets [141], in a few isolated cases islet calcification and fibrosis have been described [155]. The exocrine parenchyma displays acinar cell atrophy and some mild interstitial fibrosis [141,144].

TYPE 2 DIABETES MELLITUS Type 2 diabetes mellitus accounts for >90% of cases of diabetes [137] and afflicts individuals with insulin resistance, who generally have relative rather than absolute insulin deficiency. Patients are usually adults over the age of 40 yr with some degree of obesity and they do not require insulin to survive. However, over time their insulin secretory capacity tends to deteriorate and insulin treatment may become necessary to achieve optimal glucose control [138].

At the onset of type 2 diabetes there are no specific gross changes of the pancreas. Histologically, the islet cell mass is normal or somewhat increased. Because patients tend to live for approx 10–20 yr after the diagnosis of diabetes, the majority of pancreata examined at autopsy are from old patients with longstanding type 2 diabetes. After a long period of type 2 diabetes, there is a considerable atrophy of the pancreas with a loss of 1–40% of the total weight. Histologically, there is little or no evidence of islet cell hyperplasia or neoformation but rather a β-cell loss. At variance with what has been observed in type 1 diabetes, islet β cells are reduced at most by values of up to 50% [156]. The characteristic islet alteration in older patients with longstanding type 2 diabetes is amyloidosis [157], formerly called "hyalinization" [158]. Islet amyloid is composed of an amorphous acellular material that appears between the islet cells and the intrainsular capillaries but in advanced stages may also form globules that replace the islet cells. Typically, islet amyloid derives from amylin (also known as IAPP) deposition [159]. IAPP, a 37-amino-acid peptide showing a close relationship with the calcitonin gene-related peptide [160], is synthesized by β cells and costored in secretory granules together with insulin [161]. It is not clear whether islet amyloid represents a primary or secondary event in the pathogenesis of type 2 dia-

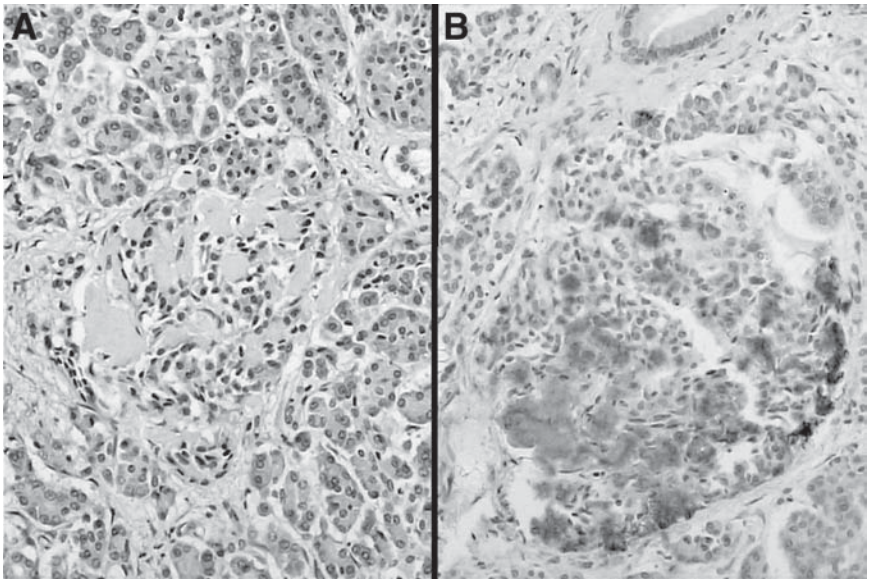

Figure 6 Pancreatic tissue from an elderly patient with longstanding type 2 diabetes. H&E stain shows amorphous globules of amyloid that replace islet cells (**A**). Typically, islet amyloid derives from amylin as demonstrated by immunohistochemical staining with anti-amylin antibody (**B**).

betes but, at any rate, it negatively interferes with islet cell function. Amyloid, which appears as a eosinophilic amorphous deposition in hematoxylin and eosin (H&E)-stained sections, is Congo red positive and shows the typical birefringence in polarized light. Amyloid can also be identified using specific antibodies directed against the IAPP molecule (Fig. 6).

PROLIFERATIVE PATHOLOGY

Proliferative pathology of the endocrine pancreas includes three disorders: islet hyperplasia, nesidioblastosis, and islet dysplasia [162]. Although islet hyperplasia and nesidioblastosis may coexist in the same pancreas, the two lesions can be observed independently.

ISLET HYPERPLASIA Islet hyperplasia represents an increase in islet mass resulting from an increase in islet size, number, or both. The volume density of the endocrine component of the pancreas is clearly in excess when compared with the corresponding values for age-matched controls. Generally islet size is >250 μm in diameter (normally it is about 225 μm).

Islet hyperplasia has been sporadically reported in asymptomatic subjects, in patients with α_1-antitrypsin deficiency [163], or associated with hyperfunctional syndromes such as hyperinsulinism and Zollinger–Ellison and Verner–Morrison syndromes [164–166]. However, the association between islet hyperplasia and either Zollinger–Ellison or Verner–Morrison syndrome has been questioned as no gastrin or vasoactive intestinal peptide (VIP) has been demonstrated in such hyperplastic islets [167, 168]. Islet hyperplasia has also been reported in neonates with maternal diabetes, erythroblastosis fetalis, acquired immunodeficiency syndrome (AIDS) [169], or with complex genetic or malformative syndromes such as Simpson–Golabi–Behmel syndrome [170], hereditary tyrosinemia of hepatorenal type [171], Zellweger's cerebrohepatorenal syndrome [172], leprechaunism [173], and Beckwith–Wiedemann syndrome [174].

Histologically, islet cell hyperplasia is characterized by abnormally large and apparently confluent islets, grouped in the center of the lobules. The normal distribution of the four islet cell types is retained. In addition to the increased size and number of islets, hypertrophy of β cells may be present in all hypoglycemic conditions, although it is less prominent than in neonatal nesidioblastosis. True islet hyperplasia should be distinguished from the islet crowding observed in chronic pancreatitis. In this condition the apparent increase in islet number results from atrophy of the exocrine parenchyma rather than from a real active islet proliferation [162].

NESIDIOBLASTOSIS The term nesidioblastosis was coined to designate endocrine clusters connected with pancreatic ductules indicating insular neogenesis from ductular cells [175]. This lesion, not associated with endocrine dysfunction, can be detected in normal newborn pancreas or in chronic pancreatitis. However, the term nesidioblastosis is also used to indicate the morphologic lesions associated with an endocrine disease denominated persistent hyperinsulinemic hypoglycemia (PHH), although ductulo–insular neogenesis is *per se* neither an obligatory finding nor a diagnostic or pathogenetic clue to the disease.

Persistent Hyperinsulinemic Hypoglycemia in Infancy (PHHI) PHHI is the most important form of congenital hyperinsulinism [176,177]. The clinical features of PHHI, which appear during the first days of the life, include ataxia, seizure, and coma, and the diagnosis of PHHI is based on the demonstration of a persistent insulin secretion inappropriate for the glucose concentration [176]. Therapeutic approaches include glucose infusions, diazoxide or octreotide therapy, and, sometimes, subtotal pancreatectomy [178–180]. Physiologic and morphologic studies have recently indicated that PHHI is a hyperfunctional β-cell disorder associated with different pathologic changes [181]. In addition, molecular studies have dem-

onstrated that PHHI results from at least three different genetic defects: (1) The first are mutations in the two subunits *SUR1* and *KIR6.2* genes of the sulfonylurea receptor (SUR) at locus 11p15.1 [182–184]. The SUR protein, which is closely associated with ATP-sensitive potassium (K_{ATP}) channels in β cell membranes, regulates insulin secretion through the modulation of potassium and, indirectly, calcium channels. (2) The second are mutations of genes encoding for enzymes such as glucokinase and glutamate dehydrogenase, which regulate the rate of insulin secretion. (3) The third is loss of heterozygosity (LOH) at region 11p15.1 of maternal alleles unmasking paternally inherited recessive *SUR1* or *KIR6.2* mutations [179,185,186].

Several studies have indicated that there are two forms of PHHI: one characterized by focal adenomatous hyperplasia (focal PHHI) and one characterized by a diffuse β cell abnormality (diffuse PHHI) [187–190]. The distinction between these two forms is important from a therapeutical point of view because infants suffering from the focal form may be cured by partial pancreatectomy [191].

Focal PHHI Focal PHHI is found in a quarter to a half of all cases of PHHI [124,185]. Macroscopically, the pancreas has a normal appearance. Usually the lesion is unifocal [124] and it has been located either in the head and body [179] or in the body and tail of the pancreas [181]. Focal lesions require systematic analysis of serial sections of all available pancreatic tissue to be detected. Histologically, there is an accumulation of islet cell clusters, which are separated by thin rims of acinar cells or strands of connective tissue. Occasionally they may be attached to small ducts forming ductulo–insular complexes. Some cells are large and display hypertrophic nuclei and the proliferation rate seems to be increased. By using immunohistochemistry islet-like clusters appear to be composed of all four islet cell types; however, β cells are more numerous than in normal islets, representing 70–90% of all endocrine cells [181]. β cells are large, strongly immunoreactive for proinsulin, and show an ultrastructural pattern of functional hyperactivity. The islets outside the focus show endocrine cells with normal size, appearance, and distribution.

Diffuse PHHI Diffuse nesidioblastosis is the most frequent proliferative lesion associated with PHHI. As in focal PHHI, the pancreas displays no gross abnormalities. Histologically, nesidioblastosis involves diffusely the tail and the body, while the head is less frequently affected. The key lesions are β cell hypertrophy as evidenced by nuclear enlargement [192], prominent ductulo–insular complexes, abundant poorly defined endocrine cell clusters (some large) with often irregular outlines, and islets of variable size [181,187,188,193,194]. Morphometric studies revealed an increased nuclear volume of β cells in comparison to age-matched controls. A tetraploid pattern of DNA content has been found in enlarged β cell nuclei. Immunohistochemical investigations have found a tendency for an increased number of β cells and a decrease in δ cells, with an increased β to δ cell ratio [189,195]. In a minority of patients, routine histology, despite systematic investigation of serial sections, fails to reveal clear-cut diagnostic lesions. Immunohistochemical analysis may disclose subtle differences from age-matched controls [196]. The most significant finding is a widespread dissemination of individual endocrine cells or small endocrine cell clusters throughout the exocrine tissue, mimicking the morphology of a perinatal pancreas. Morphometric studies have demonstrated that in the majority of patients the proportion of endocrine tissue or insular cells is not significantly increased [187]. Although a reduced number of α and δ cells has been reported in some investigations [195,197], this finding has not been confirmed in other studies [188].

Persistent Hyperinsulinemic Hypoglycemia in Adults (PHHA) PHHA is a very rare cause of persistent hyperinsulinemia and hypoglycemia not associated with insulinoma. About 40 cases have been reported in the world literature [124,198–205]. There is a female predominance, and the onset of symptoms occurs in middle age. The duration of hypoglycemic symptoms is highly variable, ranging from a few days to 18 yr before the pancreatectomy, which had been performed on all patients reported. Elevated blood insulin levels during fasting and hypoglycemia in the absence of an insulin-secreting tumor are indicative of PHHA. To exclude the presence of a small insulinoma a complete and careful sampling of the pancreas with systematic histologic investigation should be performed. Unlike pediatric patients with PHHI, mutations in the sulfonylurea receptor have not been detected in the small number of adults with PHHA investigated for such genetic alterations [206].

Histologically, the most characteristic finding is represented by the presence of endocrine cells, mainly β and α cells, scattered as single elements or small clusters (Fig. 7) throughout the exocrine parenchyma in direct connection with or in close apposition to ductules. Islets are variably increased in size and some of them are localized in the connective tissue surrounding the interlobular ducts, a pattern that is seen in the fetus but not in the normal adult pancreas [162].

ISLET DYSPLASIA Islet dysplasia is a lesion with still uncertain proliferative significance and questionable potential, characterized by (1) islets of normal or slightly increased size showing structural abnormality often with trabecular appearance; (2) loss of the normal topographic and quantitative relationship between the four main islet cells with sharp prevalence of one type; and (3) mild cellular atypia. If the lesion reaches 0.5 mm in size, it should be considered a microadenoma. Dysplastic islets are frequently found in pancreas specimens from patients with multiple endocrine neoplasia type 1 (MEN1) syndrome [207]. This finding suggests that endocrine tumorigenesis in MEN 1 pancreas follows subsequent steps including hyperplastic and dysplastic changes. This view is also supported by some experimental evidence of endocrine pancreatic tumorigenesis in transgenic mice [208–211].

ENDOCRINE TUMORS OF THE PANCREAS

ORIGIN AND CLASSIFICATION The cells of origin of pancreatic endocrine tumors (PETs) are virtually all those forming the endocrine part of the pancreas. These cells are located both in the islets and in the epithelium of ducts and ductules. Ductule cells are considered to be multipotent and play a significant role in pancreas regeneration. Experimental models in animals have shown that regeneration of pancreatic parenchyma after partial pancreatectomy begins either from preexisting differentiated exocrine and endocrine cells or from regenerating ductules that give origin to new pancreatic lobules and islets

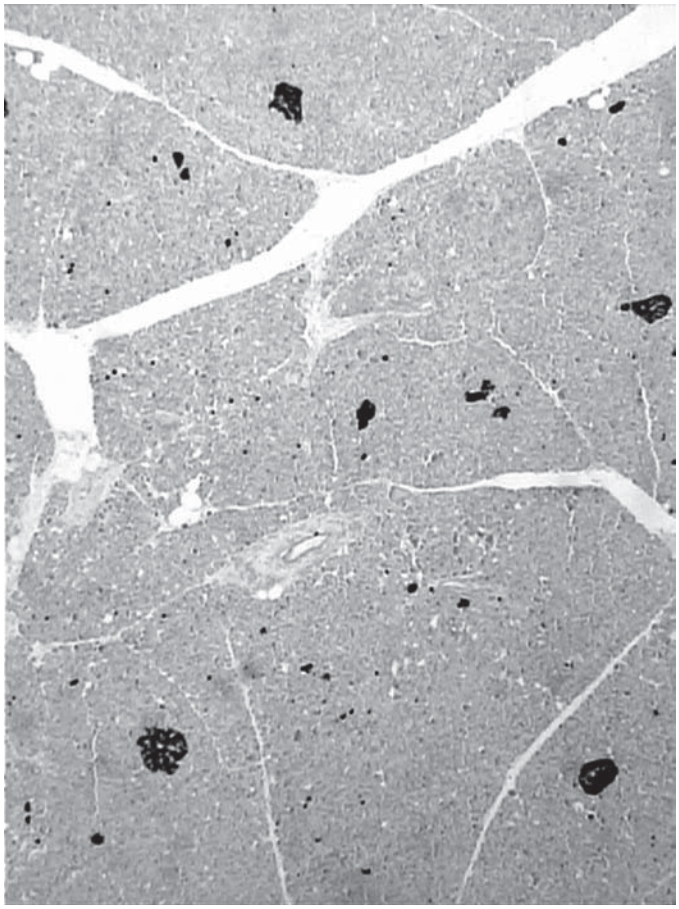

Figure 7 Pancreatic tissue from an adult patient with PHHA. β Cells positive for insulin are present both in the islets and as single elements or small clusters throughout the exocrine parenchyma.

Table 5
Number of Patients Alive with Metastatic Disease or Died of Disease in Relation to Ki-67 Proliferative Index from Different Series of Well Differentiated Pancreatic Endocrine Tumors

Ki-67 ≤ 2%	*2%<Ki-67 < 5%*	*Ki-67 > 5%*	*References*
0/19	6/8	11/17	[230]
0/20	2/8	20/23	[233]
0/6	1/10	3/13	[236]
—[a]	8/21	12/17	[235]
0/45	17/47 (36%)	46/73 (63%)	

[a] The follow-up of patients with Ki-67 index <2% was not reported.

[212]. Interestingly, insulin-like growth factor-1 (IGF-1) is abundantly expressed in the loose connective matrix surrounding the newly formed ductules [213].

Histological patterns similar to those found in the experimental regeneration of the pancreas, with emphasis on prominent nesidioblastosis, have been detected in human pathologic samples in association with pancreatitis, cystic fibrosis, and ductal adenocarcinoma [214–216]. On the basis of these findings, it has been suggested that multipotent ductular cells are possible cells of origin of PETs. However, ploidy studies have demonstrated that, unlike PETs, nesidioblastosis is a fundamentally euploid, nondysplastic growth. As a consequence, nesidioblastosis associated with PETs may be a consequence of the trophic action of hormones or growth factors produced by tumor cells [217].

Experimental models represented by transgenic mice developing heritable PETs indicate that PETs originate from the transformation of intrainsular mature cells rather than from ductule cells [218]. In MEN 1 pancreas, moreover, multiple lesions associated with tumors point to a complex multistep process involving well-differentiated islet cell types [219,220]. All subsequent steps in tumorigenesis found in human MEN 1 pancreas and including intrainsular hyperplastic–dysplastic lesions, monotypic multiple microadenomas, multitypic macroadenomas, and carcinomas with eutopic and ectopic cell populations have also been found in the MEN 1 tumor suppressor mouse knockout model [221].

PETs represent a heterogeneous group of neoplasms showing different morphological, clinical, and molecular features. Since the beginning of the last century, when the first report of a tumor believed to originate from the endocrine pancreas was published [222], several investigators have tried to elucidate the clinicopathological and molecular characteristics of these neoplasms. Because different methodological approaches have been used to classify these tumors, a variety of nomenclatures have been proposed, which have often created confusion among pathologists and clinicians [223]. In 1995, a group of endocrine pathologists [224] proposed a revised classification of neuroendocrine tumors of the lung, gut, and pancreas. The purpose of this classification was to identify clinical and morphological features that were helpful in delineating categories of tumors with different prognoses. Among prognostic parameters, proliferative markers appeared to be promising and useful in recognizing tumors with a high risk of malignancy and poorer outcome [225–228]. The first report indicating the utility of a proliferative marker in predicting the malignancy of PETs was published in 1992 by Pelosi et al. [229]. These authors demonstrated that a proliferating cell nuclear antigen (PCNA) index higher than 5% correlated with a decreased mean survival of patients. However, the Ki-67 index, evaluated using the monoclonal antibody MIB1, has emerged from different studies to be better than the PCNA proliferative rate in predicting patient outcome [230–237]. It has been suggested that a Ki-67 proliferative index of <2% is indicative of benign behavior [230]. The risk of malignancy increases progressively from a Ki67 index of 2% to >10% (Table 5) (Fig. 8). However, in addition to the Ki-67 index, a number of morphological parameters have been investigated and proved to be useful as behavior-predicting variables for patients with endocrine tumors of the pancreas [220,230]. The following histopathological criteria of malignancy should be considered: tumor size (larger tumors are more aggressive), invasion of nearby tissue, structural atypia with prevalence of broad solid areas, presence of necrosis, cellular atypia with increased nuclear/cytoplasmic ratio, irregular dis-

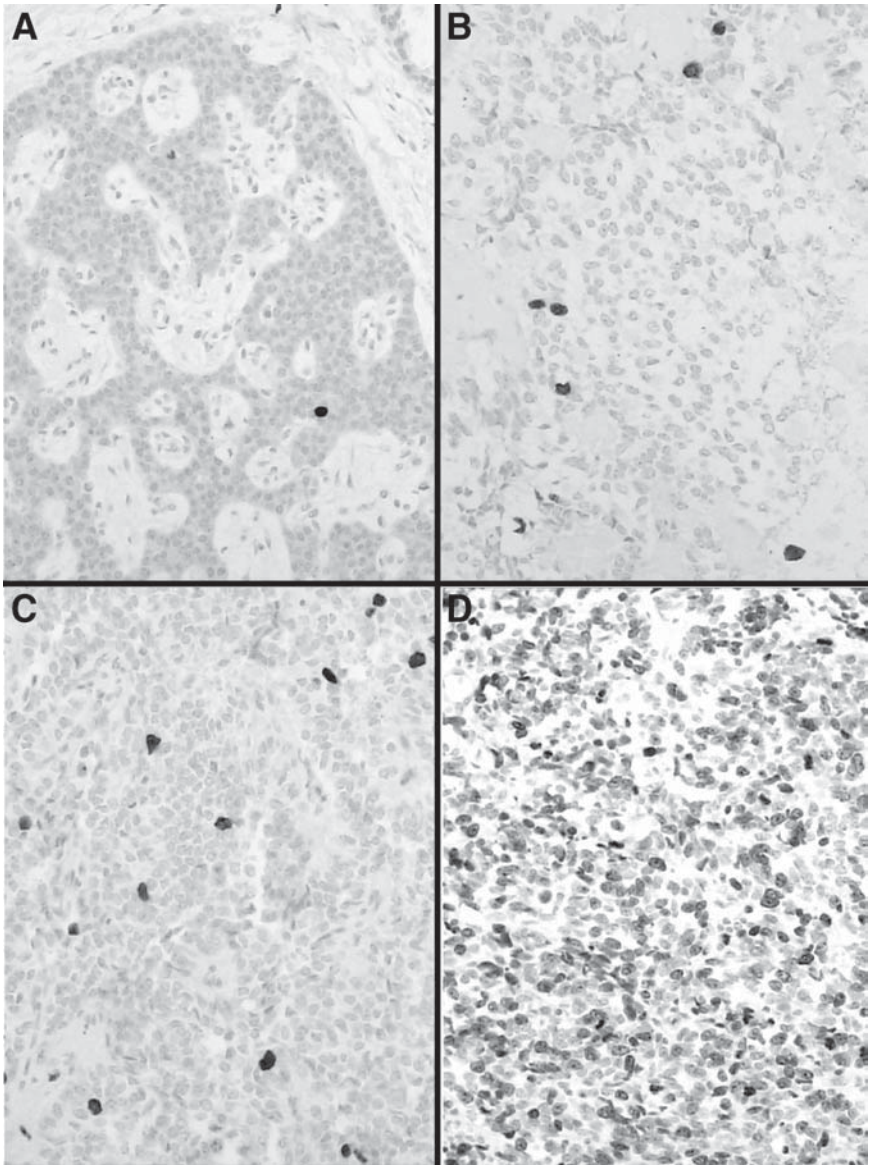

Figure 8 Ki-67 immunoreactivity in different types of pancreatic endocrine tumors. The Ki-67 proliferative index increases progressively from benign tumors (**A**) to poorly differentiated endocrine carcinomas (**D**). Tumors with uncertain behavior (**B**) and well differentiated endocrine carcinomas (**C**) show an intermediated Ki-67 proliferative index.

tribution of chromatin and evident nucleoli, and more that two mitoses per 10 high power field (HPF). Among these criteria, vascular microinvasion seems to be the more sensitive and specific in predicting malignancy [230]. However, vascular microinvasion should be accepted as true only when the vessel wall is identified through CD31 or factor VIII–related antigen immunostaining [238] (Fig. 9). The criteria proposed by the international group of pathologists in 1995 represented the basis for the recent WHO classification of pancreatic endocrine tumors (Table 6) [239]. Although the practical value of the new WHO classification remains to be determined with studies on large series from different institutions, some recently published papers have indicated the utility and reproducibility of such a classification [240,241].

Endocrine tumors of the pancreas are classified according to the tumor cell type or the clinical status of the patient with or without association with a tumor-derived hyperfunctional syndrome. The latter approach identifies two broad categories of functioning or nonfunctioning tumors.

Immunohistochemical cell typing of PETs may provide morphofunctional information; however, such data need to be correlated with levels of circulating hormones and patient clinical symptoms. On careful investigation most tumors prove to be composed of different cell types, while, in general, only one cell type proves to be responsible for the associated hyperfunctional syndrome, if present. Indeed, well-differentiated PETs are often associated with hyperfunctional syndromes determining their specific clinicopathological profile. In such cases the

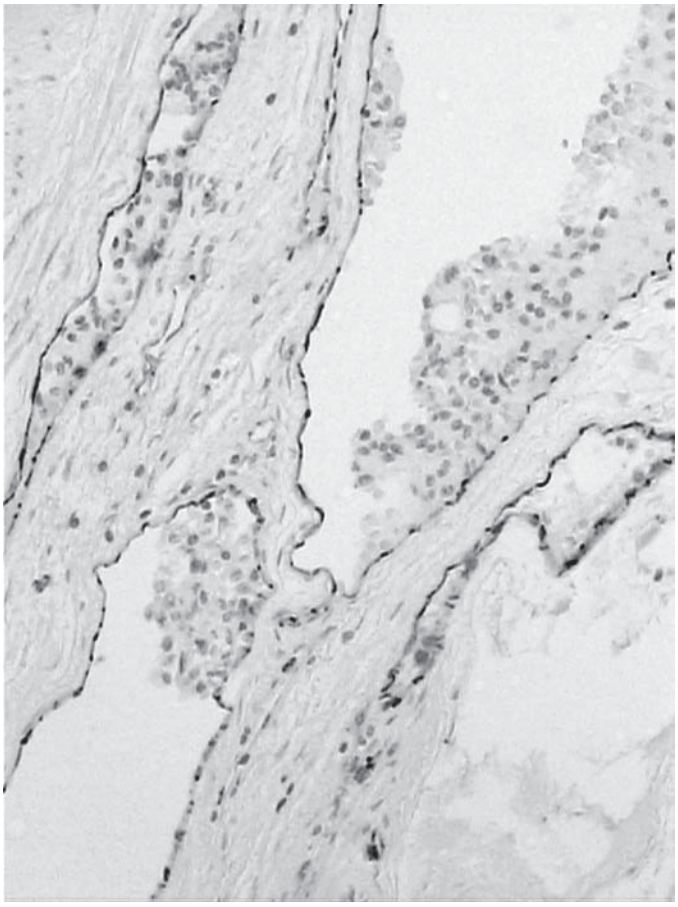

Figure 9 Angioinvasion in a well differentiated endocrine tumor of the pancreas with uncertain behavior. Vessels are identified using antibodies directed against CD31.

Table 6
WHO Classification of Pancreatic Endocrine Tumors [239]

1	Well differentiated endocrine tumor
1.1	Benign behavior: confined to the pancreas, nonangioinvasive, <2 cm in size; ≤ two mitoses and ≤ 2% Ki-67-positive cells per 10 HPF
1.1.1	Functioning: insulinoma
1.1.2	Nonfunctioning
1.2	Uncertain behavior: confined to the pancreas, ≥ 2 cm in size, more than two mitoses and > 2% Ki-67-positive cells per 10 HPF, or angioinvasive
1.2.1	Functioning: gastrinoma, insulinoma, VIPoma, glucagonoma, somatostatinoma, inappropriate hormone secreting tumors[a]
1.2.2	Nonfunctioning
2	Well differentiated endocrine carcinoma
2.1	Low-grade malignant: gross local invasion and/or metastases
2.1.1	Functioning: gastrinoma, insulinoma, glucagonoma, VIPoma, somatostatinoma, inappropriate hormone secreting tumors[a]
2.1.2	Nonfunctioning
3	Poorly differentiated endocrine carcinoma High-grade malignant: small to large cell carcinomas

[a] Inappropriate hormone secreting tumors may cause the following endocrine syndromes: Cushing (ACTH), acromegaly or gigantism (GRF), hypercalcemia, and so forth.

Table 7
Functional Classification of Pancreatic Endocrine Tumors

%	*Type*	*Behavior*
5–10	Nonfunctioning, clinically silent	Mostly benign
50	Insulinomas	90% benign
20	Other functioning tumors: gastrinoma, glucagonoma, somatostatinoma, carcinoid, Cushing's tumors, and so forth	50–90% Malignant, low grade
20	Nonfunctioning, locally symptomatic tumors	70–80% Low grade malignancies
1–5	Small cell carcinoma with poor endocrine differentiation	All malignant, high grade

tumor itself may be denominated according to the associated syndrome as "insulinoma," "gastrinoma," and so forth. Remarkably, the tumor-associated hyperfunctional syndrome is *per se* more indicative of the tumor behavior than the morphologic cell typing (Table 7).

PETs are often not associated with specific hormone-dependent clinical symptoms (nonfunctioning tumors) and present either with tumor mass symptoms or as an incidental finding. The use of syndrome-associated tumor denomination (with desinence in "oma") should be avoided for PETs lacking hyperfunctional syndromes in spite of identification of specific functional cell types. It is recommended that such growths may be denominated as "nonfunctioning PET mainly composed of a specific cell type" (i.e., "nonfunctioning PET mainly composed of somatostatin-producing δ cells" instead of "somatostatinoma of the pancreas"). In general, the identification of a specific hormone cell content in a nonfunctioning PET is poorly predictive of the tumor behavior.

The general histological classification of PETs comprises the two major categories of well differentiated and poorly differentiated PETs. *Well-differentiated PETs* (*WDETs*) are characterized by tumor cell monomorphism, absent or mild nuclear atypia, and low mitotic and proliferative status together with the frequently observed trabecular structure. As a rule, WDETs confined to the pancreas that are nonangioinvasive, show two or fewer mitoses per 10 HPF, ≤ 2% Ki-67 positive cells, and are <2 cm in diameter follow a benign course (macroadenomas). WDETs confined to the pancreas but showing angioinvasion and/or perineural invasion, or more than two mitoses per 10 HPF, or > 2% Ki-67-positive cells are at increased risk for malignant behavior (uncertain behavior). *Well differentiated endocrine carcinomas* (*WDECs*) are epithelial growths that are locally invasive or with evidence of metastases to local lymph nodes or to the liver. Most tumors are 3 cm or more in size (mean 5–6 cm) when diagnosed. Structurally they are formed by solid nests, trabeculae, or larger cell aggregates. Moderate atypia with fairly prominent nucleoli and nuclear hyperchromatism is often, but not always, seen in tumor cells displaying an increased number of mitoses (two to nine per 10 HPF) or Ki-67 proliferative index (2–10%) and, more important, angioin-

vasion or perineural invasion. *Poorly differentiated endocrine carcinomas* (*PDECs*) show a mostly solid structure either organized in large, poorly defined aggregates often with central necrosis, or diffuse sheets of cells with multiple minute foci of necrosis. PDEC cells are highly atypical, small to intermediate and, in some cases, large in size, showing more than 10 mitoses/10 HPF, more than 10% Ki-67-positive cells, prominent angioinvasion, and frequent p53 nuclear accumulation at immunohistochemistry. PDECs are highly invasive, invariably presenting with distant metastases to the liver and other organs, often in extraabdominal sites.

WDETs must be distinguished from solid-pseudopapillary tumors, acinar cell carcinomas, and pancreatoblastomas. WDETs may mimic a solid-pseudopapillary tumor but the reactivity for endocrine granule stains (Grimelius, chromogranins) or for hormones supports the diagnosis of an endocrine tumor, whereas strong immunostaining for α_1-antitrypsin favors a solid-pseudopapillary neoplasm [220]. It is worth recalling that NSE reactivity also occurs in solid-pseudopapillary tumors so that it cannot be used as differential diagnostic marker [242]. Distinguishing WDETs from acinar cell carcinomas is very important because of the worse prognosis of the latter. The presence of an even restricted area of acinar differentiation or larger tumor cells with large nuclei, prominent nucleoli, abundant cytoplasm, periodic acid-Schiff (PAS)-positive granules, positive immunostaining for trypsin and lipase, and the lack of immunoreactivity for chromogranins, synaptophysin, and hormones strongly favors the diagnosis of acinar cell carcinoma [220,243]. Age represents an important criterion in distinguishing WDETs from pancreatoblastomas as the former are very rare in the first decade of life. The presence of squamoid nests, an acinar structure, and reactivity for α-fetoprotein supports the diagnosis of pancreatoblastoma [242,244].

WELL-DIFFERENTIATED FUNCTIONING ENDOCRINE TUMORS

Insulinoma Insulinoma is the most common type of functioning PET [220,245–247] and the incidence has been estimated to be $1/1.25 \times 10^6$ persons [248]. Although insulinomas can occur at any age, they are more frequent between 30 and 60 yr of age. Children under 15 are rarely affected [249,250]. Up to 90% of insulinomas are benign solitary tumors suitable for surgical resection or enucleation [251] whereas malignant tumors are present in only 5–10% of cases [252]. Clinically, patients present the well-known symptoms associated with hypoglycemia, especially after periods of fasting. Headache, weakness, dizziness, dysarthria, incoherence, convulsion, and coma represent the most common symptoms which are due to the deleterious effects of hypoglycemia on brain function [253].

Insulinomas occur in any part of the pancreas [254], but they are more frequent in the body–tail region [220]. The tumor is single in the majority of cases although multiple nodules may coexist, a finding that must suggest the presence of MEN 1 syndrome [220,255]. Insulinomas are usually small with an average diameter of about 1.5 cm. Interestingly, there is no relationship between the size and the severity of clinical symptoms. Insulinomas are well circumscribed, at least partially encapsulated, with a color that can vary from gray-white to deep red (Fig. 10).

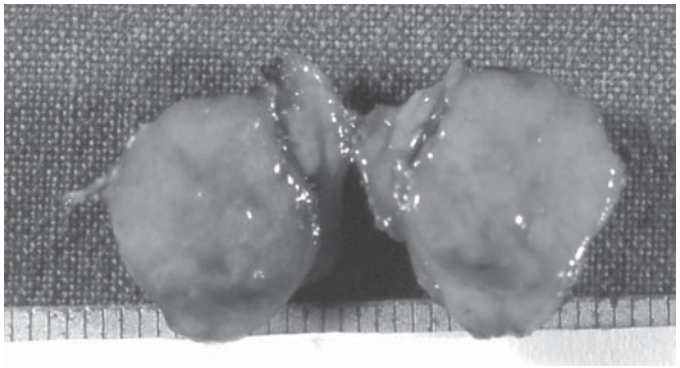

Figure 10 Gross appearance of an insulinoma of the pancreatic body. The tumor was enucleated and is well circumscribed. (Color illustration appears in insert following p. 148.)

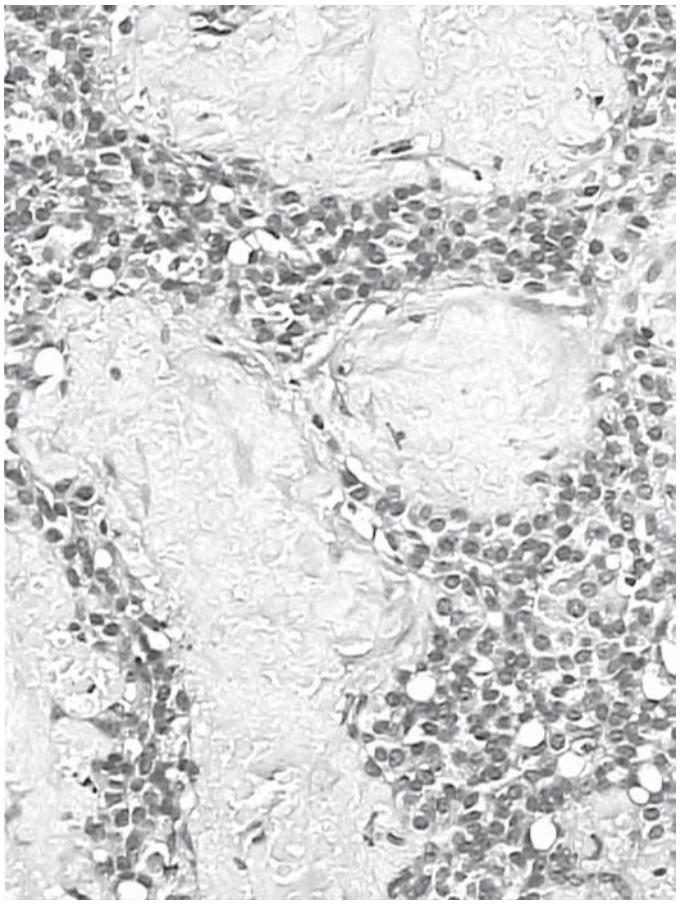

Figure 11 Microscopic feature of the insulinoma shown in Fig. 10. Cells are uniform with no or mild atypia and form trabecular structures separated by abundant amorpohous globules of amyloid.

Histologically, insulinomas may show different architectural patterns including trabecular–gyriform, lobular, and solid structures. A peculiar histologic finding observed in insulinomas is the presence of amyloid in the fibrovascular stroma, in close proximity to tumor cells (Fig. 11). Such deposits show the typical green birefringence after staining with Congo red and examination in polarized light. As in the islets of type 2 diabetic patients, the amyloid of insulinomas contains amylin (IAPP) [256]. Tumor nuclei in cases with benign behavior are round to ovoid with finely stippled chromatin and inconspicuous nucleoli.

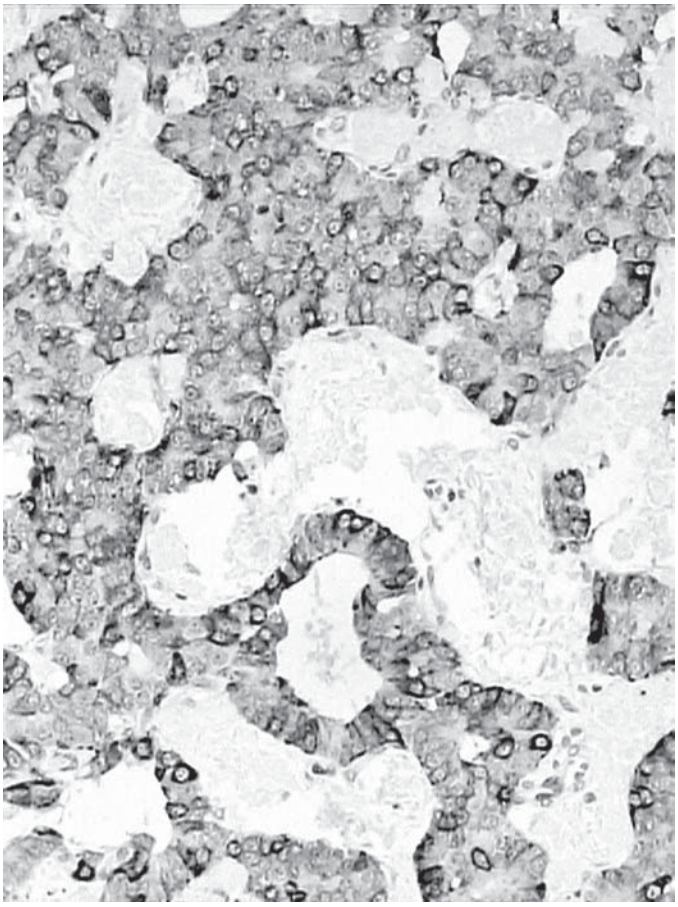

Figure 12 Insulin immunoreactivity in a pancreatic insulinoma. Cells are positive, whereas amyloid depositions are completely negative.

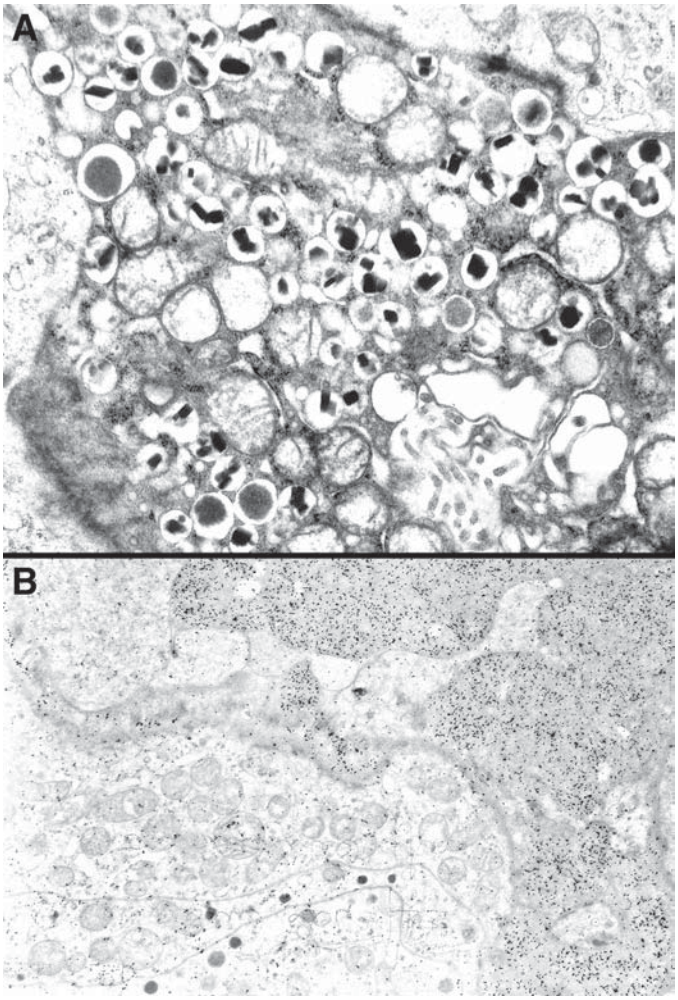

Figure 13 Ultrastructural features of a pancreatic insulinoma. Tumor cells contain numerous typical crystalline type secretory granules (**A**). **B** is an example of the immunogold technique showing that amyloid deposits are positive for amylin.

Immunohistochemically, almost all insulinomas are positive for insulin (Fig. 12) and proinsulin [257] as well as for amylin [257, 258]. About half of insulinomas are multihormonal and cells positive for glucagon, somatostatin, PP, gastrin, adrenocorticotropic hormone (ACTH), and calcitonin have been described [257–259]. In addition to hormone peptides, insulinomas express the prohormone convertases PC2 and PC3, the carboxypeptidase H (CPH) and 7B2, which play a role in the proteolytic conversion of proinsulin to insulin [260]. Malignant insulinomas have been found to be immunohistochemically positive for *TGF*α, K-*ras*, N-*ras*, and *p53*, and point mutations at codon 12 of the K-*ras* gene have been also demonstrated [261].

Ultrastructurally, three main types of cells have been described: (1) densely granulated cells with typical crystalline-type β granules (Fig. 13); (2) cells with solid, round to slightly pleomorphic granules; and (3) sparsely granulated cells [262, 263]. According to Berger et al. [262] densely granulated tumors (type A) are functionally characterized by a good response to diazoxide and somatostain treatment. In contrast, sparsely granulated tumors (type B) usually do not respond to diazoxide or somatostatin.

About 90–95% of insulinomas are benign at the time of diagnosis. Malignant insulinomas can be unquestionably identified only in the presence of metastases and/or gross local invasion. Malignant insulinomas often grow slowly, with a median survival of patients of 4 yr.

Glucagonoma Glucagonoma is a WDET of the pancreas with α-cell differentiation causing a typical endocrine syndrome characterized by dermatitis (necrolytic migratory erythema), stomatitis, diabetes, weight loss, and anemia due to excess of glucagon [253]. Glucagonomas are rare tumors representing approx 8% of functioning neoplasms and 5% of all clinically relevant pancreatic endocrine tumors [220], with an annual incidence of 0.01–0.1 cases per 10^6 population (Table 8). They occur most often in adult patients (average age of 55 yr) and are slightly more common in women. Glucagonomas must be distinguished from small nonfunctinoning glucagon-producing tumors that are often incidentally found at autopsy or at surgery and have

Table 8
Incidence and Malignancy Rates of Different Types of Well Differentiated Pancreatic Endocrine Tumors

Tumor type	*Incidence (new cases per 10^6 population/yr)*	*Malignancy rate*
Insulinoma	1–2	<10%
Glucagonoma	0.01–0.1	50–80%
Somatostatinoma	Unknown	70%
PP-secreting tumor	1–2	30%
VIPoma	0.05–0.2	40–70%
Gastrinoma	0.5–1.5	60–90%

Modified from Mullan et al., 2001 [270].

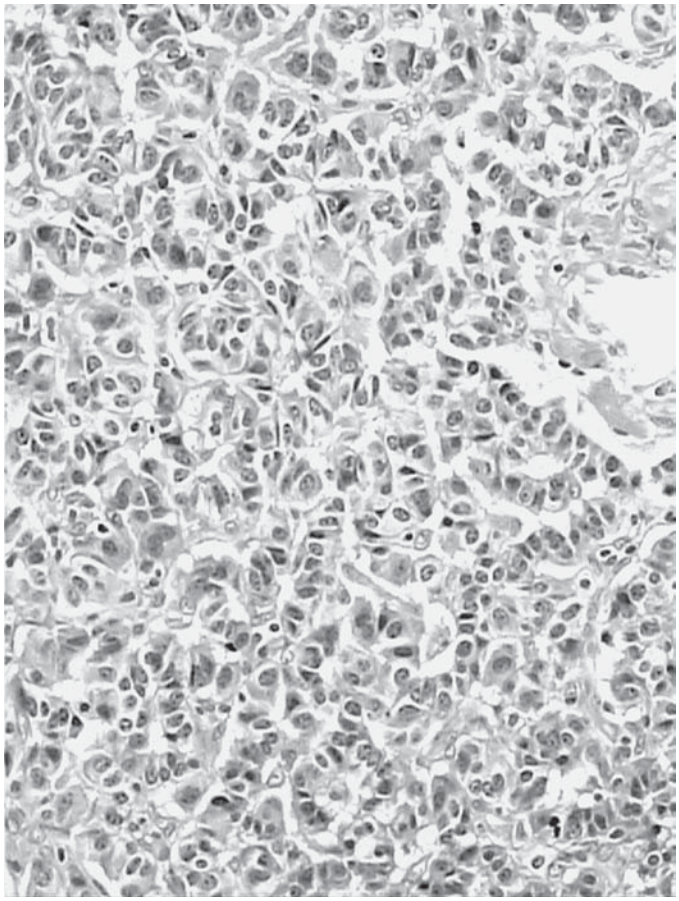

Figure 14 Glucagonoma of the pancreas. Tumor cells are well differentiated and form trabecular structures.

a benign behavior. In fact, the presence of the full-blown glucagonoma syndrome is frequently indicative of a large malignant tumor.

Most tumors are single, localized in the body–tail region, and large, with a mean diameter of 7.6 cm [264]. The majority of glucagonomas are malignant showing either local invasion or metastatic spread to the liver, regional lymph nodes, bone, peritoneum, lung, and adrenal in decreasing order [265].

Histologically, they usually show an irregular association of trabecular and diffuse patterns of growth (Fig. 14). Tumor cells are polygonal with faintly granular, often abundant, cytoplasm.

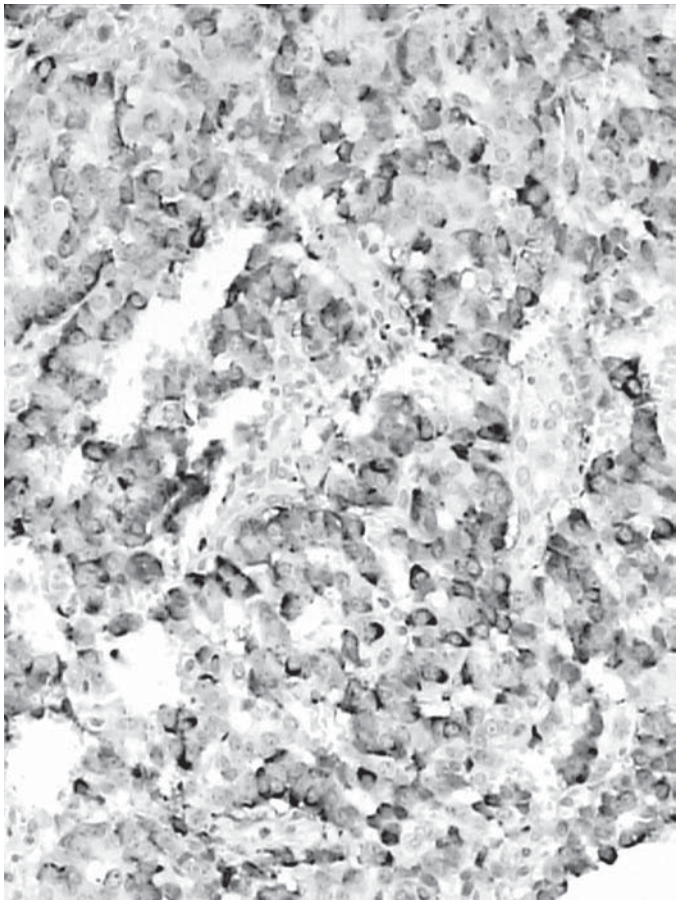

Figure 15 Glucagon immunoreactivity in a pancreatic glucagonomas.

Vascular and perineural invasion is frequently observed; mitoses and nuclear atypia are rare. Tumor cells are strongly stained with the Grimelius' silver impregnation. Immunohistochemistry reveals positivity for glucagon (Fig. 15) and for one or more peptides derived from proglucagon, such as glicentin and glucagonlike peptides (GLP)-1 and GLP-2 [266,267]. In addition, glucagonomas may also contain PP, δ, and β cells as revealed by pancreatic polypeptide, somatostatin, and insulin immunoreactivities [220,266].

At the ultrastructural level, three types of secretory granules have been identified in glucagonomas [268,269]: (1) typical A-cell granules (Table 3) (Fig. 16); (2) "atypical" or "unspecific" small to medium-sized round granules with a uniform core of varying density; and (3) medium-sized granules resembling α granules of the fetal islets.

Approximately 80% of glucagonomas are malignant and 70% are metastatic at the time of diagnosis [220]. Tumors tend to grow slowly and patients may survive for several years with the disease. Surgical resection dramatically improves the clinical picture with regression of the typical symptoms. In non-resectable tumors, chemotherapy can be used although long-standing somatostatin analogues can better reduce the glucagon secretion [270]. The presence of metastases is significantly ($p < 0.001$) correlated with poorer outcome [265]. Interestingly, in a published series it has been observed that 26% of patients with

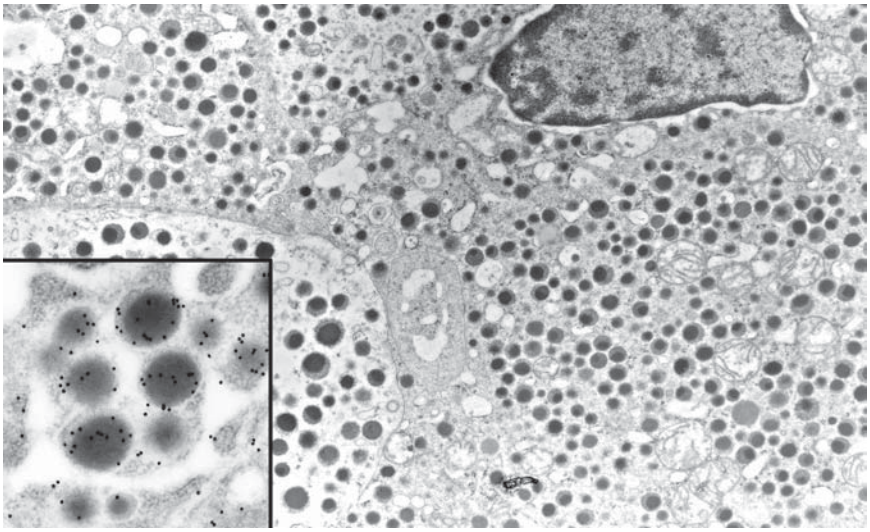

Figure 16 Ultrastructural aspect of a pancreatic glucagonoma. Tumor cells are rich in typical α-cell granules with a central or eccentrical electron-dense core surrounded by a pale halo. Glucagon immunoreactivity is restricted to secretory granules (**inset**).

Table 9
Clinical Features of Pancreatic Somatostatinomas, from Different Cases Reported in the Literature

Sex (%)		*Mean age*	*Site (%)*			*Mean Ø*		*Diar/*	*Hypo/*		*Weight*	*Abdominal*	*Liver*	*Node*
F	*M*	*(range)*	*H*	*B*	*T*	*(range)*	*Diab.*	*steator*	*achlo*	*Anemia*	*loss*	*pain*	*met.*	*met.*
63	37	55 yr (30–74)	56	12	32	6.3 cm (3–10)	96%	81%	70%	73%	85%	100%	76%	80%

H, head; B, body; T, tail; Ø, diameter (cm); Diab., diabetes mellitus; Diar/steator, diarrhea/steatorrhea; Hypo/achlo., hypo/achlorhydria; met., metastases.

glucagonoma developed a second, or even third, hyperfunctional syndrome, including the Zollinger–Ellison and hyperinsulinemic syndrome [271].

Somatostatinoma Pancreatic somatostatinoma is an uncommon endocrine tumor composed of δ cells, associated with a complex of symptoms (the somatostatinoma syndrome) caused by hypersomatostatinemia. As yet only a small number of pancreatic somatostatinomas (approx 40 cases) have been described [272–289] and their incidence is considered to be <1% of functioning PETs. In addition to the pancreas, somatostatin-producing neoplasms also occur in the duodenum, where they are more frequent than in the pancreas [220]. Pancreatic somatostatinomas prevail in females (Table 9) and arise in adults, with an average age at diagnosis of 55 yr (range 30–74). The main clinical symptoms include diabetes mellitus, cholelithiasis, diarrhea with or without steatorrhea, weight loss, hypochlorhydria, and anemia. All these clinical features depend on the inhibitory action of somatostatin on endocrine cells producing insulin, secretin, cholecystokinin, and gastrin, as well as on gastric parietal cells, pancreatic acinar cells, and intestinal absorbing cells.

Somatostatinomas are most commonly located in the head of the pancreas although they may arise anywhere in the gland. Tumors are generally large (average diameter 5–6 cm), single, well circumscribed but not encapsulated, and malignant.

Histologically, somatostatinomas show the usual histologic features observed in all pancreatic endocrine tumors, with cells forming solid sheets, trabeculae, or acinar structures (Fig. 17). Tumor cells generally show mild nuclear atypia and rare mitoses. Extensive necrosis is generally lacking but angioinvasion and perineural invasion are often found. Psammoma bodies, which are frequently observed in duodenal δ cell tumors, are rare in pancreatic tumors [289]. Amyloid deposits are infrequent [277,281] and, unlike those of insulinomas, do not react with anti-amylin antibodies [281]. Tumor cells show varying degree of immunoreactivity for somatostatin (Fig. 17). In addition, several cases show positivity for other peptides, including calcitonin, adrenocorticotropin, and gastrin [220,290].

Ultrastructurally, tumor cells show secretory granules of two types: (1) large (250–450 nm) granules with homogeneous, variably electron- dense cores, closely bound by limiting membrane, resembling those of normal δ cells and (2) smaller (150–300 nm) granules with dense cores surrounded by a thin peripheral halo.

There are several clinicopathologic differences that help to differentiate pancreatic from duodenal (ampullary) δ cell tumors, especially when the ampullary neoplasms invade the pancreatic head. Duodenal δ cell neoplasms, unlike the pancreatic ones, are generally of small size, display a typical acinar (glandular) pattern of growth with numerous psammoma bodies (Fig. 17), do not induce the classical somatostatinoma syndrome (nonfunctioning tumors), and show a relatively strong association with the von Recklinghausen disease (neurofibromatosis type 1) [291,292].

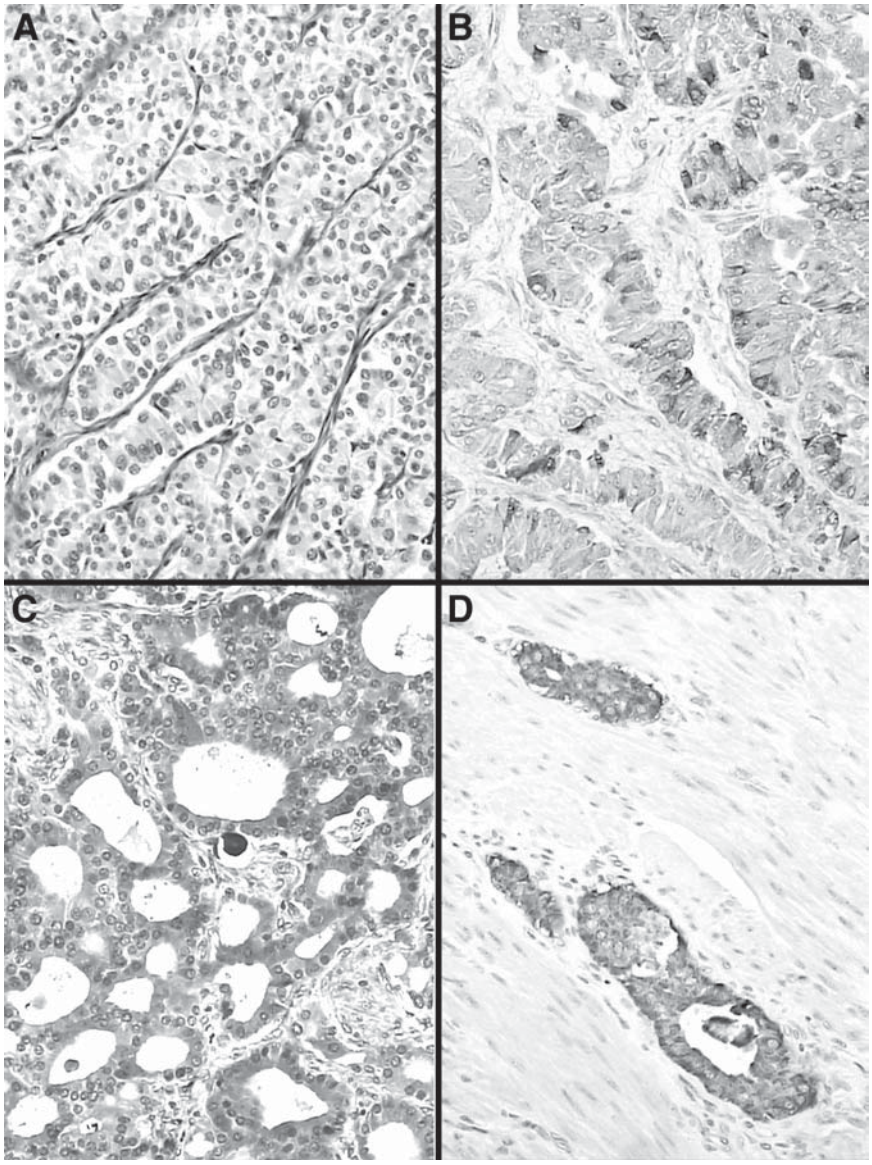

Figure 17 δ cell tumors of the pancreas (**A,B**) and duodenum (**C,D**). Pancreatic tumor shows a trabecular architecture (**A**), while the duodenal neoplasm presents the typical acinar pattern with psammomas. **B** and **D** are examples of immunostainings for somatostatin.

VIPoma VIPoma is an endocrine tumor, predominantly occurring in the pancreas, that produces the Verner–Morrison syndrome (WDHA: watery diarrhea, hypokalemia, achlorydria) due to the secretion of VIP, peptide histidine methionine (PHM), and other hormone-like substances. Pancreatic VIPomas are rare neoplasms accounting for approx 5–8% of all PETs [223,247]. In a recent review of the literature, including a total of 374 articles, VIPomas of the pancreas represented 179 of 241 (74%) neoplasms associated with the WDHA syndrome [293]. The extrapancreatic tumors causing the WDHA syndrome include neurogenic tumors such as ganglioneuroblastomas, ganglioneuromas and neuroblastomas, and epithelial endocrine neoplasms located in the lung, small bowel, and other sites. Pancreatic VIPomas show a slightly higher prevalence in females [293–295]. The average age of insurgence of pancreatic VIPomas is 50.5 yr (range 15–82 yr) and is much higher than the average age of patients with extrapancreatic neurogenic VIP-secreting tumors (average age 7.3 yr). A family history is generally absent, but an association with the MEN 1 syndrome has been found in 11.2% of the patients with pancreatic VIPomas [293].

The tumor is single in 95% of cases and is more frequently located in the tail of the gland [220,293]. The diameter ranges from 1.5 to 20 cm with an average size of approx 5 cm. Tumor size is an important, but not an absolute, criterion for distinguishing benign from malignant pancreatic VIPomas. The reported metastatic rate is 47.8% for tumors <2 cm, 50% for those with a diameter from 5 to 10 cm, and 71.4% for those larger than 10 cm (Table 10).

Histologically, pancreatic VIPomas show three main structural patterns: solid, trabecular, and tubuloacinar (Fig. 18) in

Table 10
Clinicopathologic Profile of Pancreatic VIPomas and of Nonpancreatic VIP-Secreting Tumors

	Pancreatic	*Nonpancreatic*	*p Value*
Overall number of cases	179 (74%)	62 (26%)	
Male/female ratio	84/95	26/35	Ne
Average age (range)	50.5 (15–82)	28.2 (1–74)	Ne
Clinical symptoms	99.4%	100%	NS
High serum VIP level	100%	100%	NS
Size (cm)			
0–1	9.1%	0	NS
1.1–2	11.8%	0	<0.05
2.1–5	32.7%	31.4%	NS
5.1–10	40%	51.4%	NS
>10	6.4%	17.1	NS
Metastases	56.4%	29.2%	<0.01
Liver	48.6%	4.2%	<0.01
Lymph nodes	15.6%	20.8%	NS
Bone	0.6%	6.3%	<0.01
Peritoneum	4.5%	2.1%	NS
Lung	2.8%	2.1%	NS
Adrenal	1.1%	0	NS
Metastases in relation to tumor size (cm)			
0–2	47.8%	0	0
2.1–5	38.9%	18.2%	NS
5.1–10	50%	22.2%	<0.05
>10	71.4%	66.7%	NS

Data obtained from the article by Soga and Yakuwa [293].
Ne, not evaluated; NS, not significant.

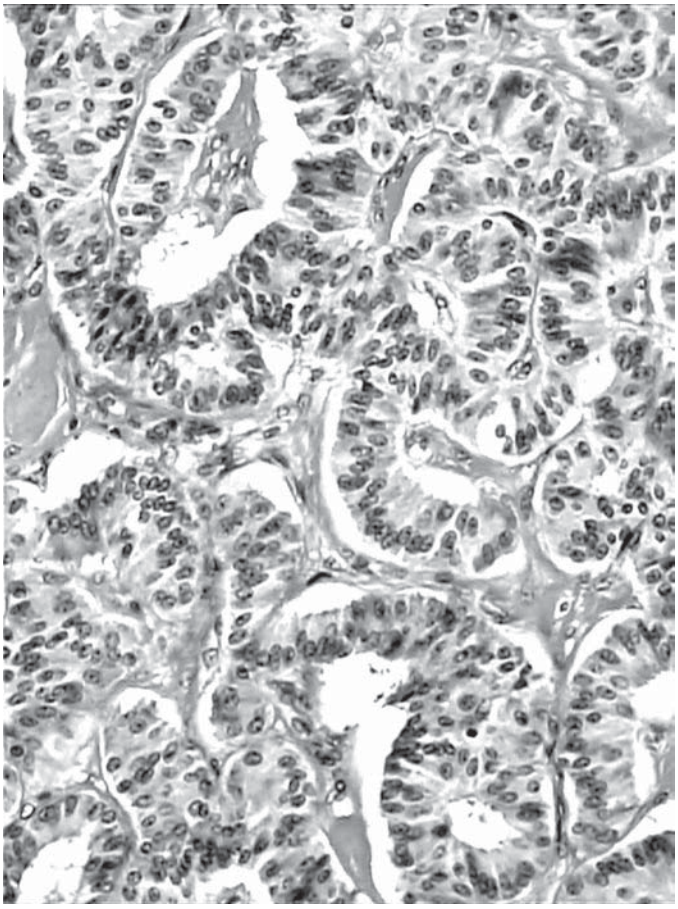

Figure 18 Pancreatic VIPoma showing a characteristic tubuloacinar pattern.

order of decreasing frequency [220]. Irregular cysts, filled with weakly eosinophilic material, sometimes interrupt the solid architecture. The cells are polygonal in shape or cylindric when they form tubules or trabeculae. Vascular and perineural invasion at the periphery of the tumor is present in 50% of cases, most of which have lymph node and/or liver metastases. The majority of neoplasms stain with Grimelius' method and are immunoreactive for general endocrine markers and VIP (Fig. 19). In addition to VIP, other hormones including peptide histidine methionine (PHM), PP, growth hormone–releasing hormone (GRF), somatostatin, and neurotensin are often immunohistochemically detected (Table 11). The frequent occurrence of PP cells in pancreatic VIPomas and the finding of both PP and VIP immunoreactivity within the same tumor cells suggest that a cell line somewhat akin to that of dorsal pancreatic PP cells might be involved in the histogenesis of these tumors [296].

Ultrastructurally, most tumors are composed of sparsely granulated (Fig. 20) or agranular cells, with a fairly developed endoplasmic reticulum and Golgi apparatus. Two types of secretory granules may be seen: (1) small, thin-haloed granules containing a moderately dense core reacting with anti-VIP antibodies; and (2) larger, more solid granules reacting with anti-PP antibodies resembling those of PP cells of PP-rich islets [220].

Up to 80% of VIPomas are reported to be metastatic at the time of diagnosis [293]. Surgery is the first choice of treatment, but in unresectable neoplasms chemotherapy and, especially, long-acting somatostatin analogues may help in controlling the clinical endocrine symptoms [297].

Gastrinoma Gastrinoma is an endocrine tumor, frequently malignant, that most often occurs in the pancreas although it can arise in extrapancreatic sites such as the duodenum, upper jejunum, and stomach [298]. This tumor type is generally associated with the Zollinger–Ellison syndrome (ZES), characterized by the presence of peptic ulcerations due to the excess of gastrin secretion by the tumor. The incidence of pancreatic gastrinomas accounts for 0.5–1.5 per year per 1 million population [270,299,300]. However, this incidence rate may be underestimated because the efficient treatment of peptic ulcers with H_2-receptor blocker drugs and proton-pump inhibitors may obscure the diagnosis of some gastrinomas that cause milder peptic ulcer disease. Gastrinomas account for about 30% of functioning PETs and are second in frequency only to insulinomas [220, 270]. There is a slightly male prevalence (male/female ratio: 3:2) and the mean age at insurgence is 38 yr (range 7–83 yr) [220]. Evidence of MEN 1 is found in 21% of patients with ZES [301].

Grossly, pancreatic gastrinomas are generally well circumscribed, but not encapsulated, tumors that are more frequently located in the head of the pancreas [220,302].

Histologically, gastrinomas may present all architectural patterns classically observed in pancreatic endocrine tumors. How-

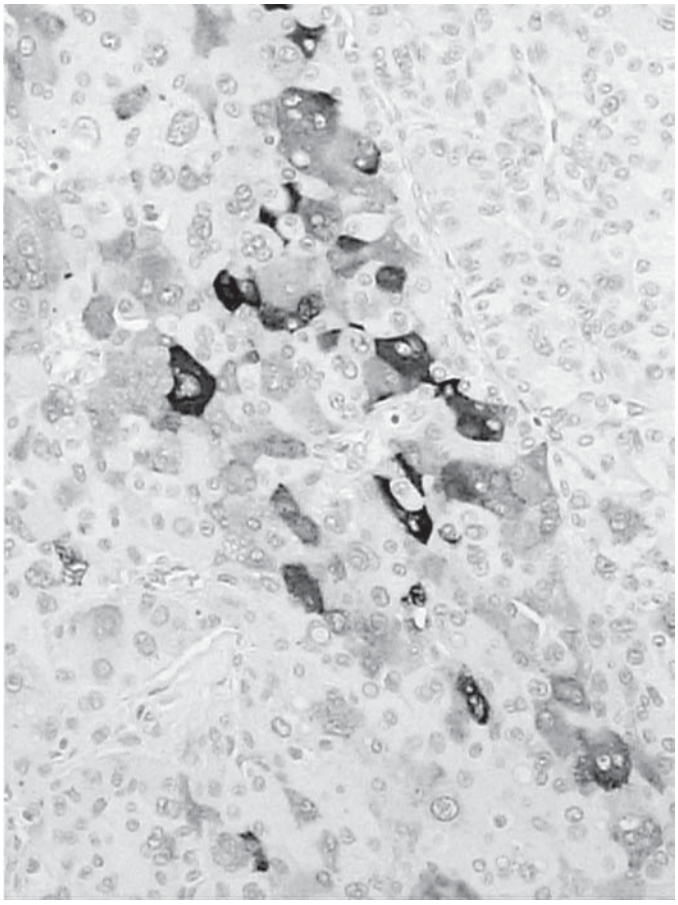

Figure 19 VIP immunoreactivity in a pancreatic VIPoma.

Table 11
Histochemical, Immunohistochemical, and Ultrastructural Profile of Pancreatic VIPomas and of Nonpancreatic VIP-Secreting Tumors

Marker	*Pancreatic*	*Nonpancreatic*	*p Value*
Grimelius	90.5%	Ne	
VIP	88.3%	100%	NS
NSE	96%	100%	NS
Neurotensin	69.2%	Ne	
Chromogranin	57.1%	1/1	
PP	53.1%	0/1	
Somatostatin	39.4%	83.3%	<0.05
Calcitonin	30.4%	1/1	
Glucagon	29.4%	0/2	
Gastrin	22.7%	0/2	
Serotonin	0	1/1	
ACTH	0	Ne	
Endocrine granules			
D_1 type	32.5%	9.1%	Ne
Other round granules	45%	63.6%	
Pleomorphic granules mixed	12.5%	0	

Data obtained from the article by Soga and Yakuwa [293].
Ne, not evaluated; NS, not significant.

ever, the most common arrangement of cells is the formation of trabeculae (Fig. 21). Tumor cells generally have round to ovoid rather uniform nuclei with slight to moderate atypia and mitoses are rarely found. In the majority of cases, it is impossible to predict, on purely histologic grounds, the malignant behavior of the neoplasm although invasion of peritumoral vessels is often found. Gastrinoma cells stain for general endocrine markers such as Grimelius' silver, chromogranins A and B, and synaptophysin. The definitive diagnosis is achieved by reactivity of tumor cells with antibodies directed against different parts of the gastrin molecule including C-terminus and non-C-terminus gastrin-17, and N-terminal gastrin-34 (Fig. 22). In addition to gastrin cells, gastrinomas may also show PP-, glucagon-, insulin-, somatostatin-, ACTH-, and serotonin-producing cells [258,303,304].

Ultrastructurally, the most characteristic secretory granules of gastrinomas are vesicular granules which resemble those of normal G cells of the pyloric mucosa.

Pancreatic gastrinomas are generally malignant. They are larger and more frequently ($p < 0.00001$) associated with liver metastases than duodenal gastrinomas [305]. Interestingly, liver but not lymph node metastases are correlated with patient survival, and the frequency of liver metastases strictly depends on the tumor size. In addition, tumors associated with the MEN 1 syndrome show a better outcome than the sporadic neoplasms [305].

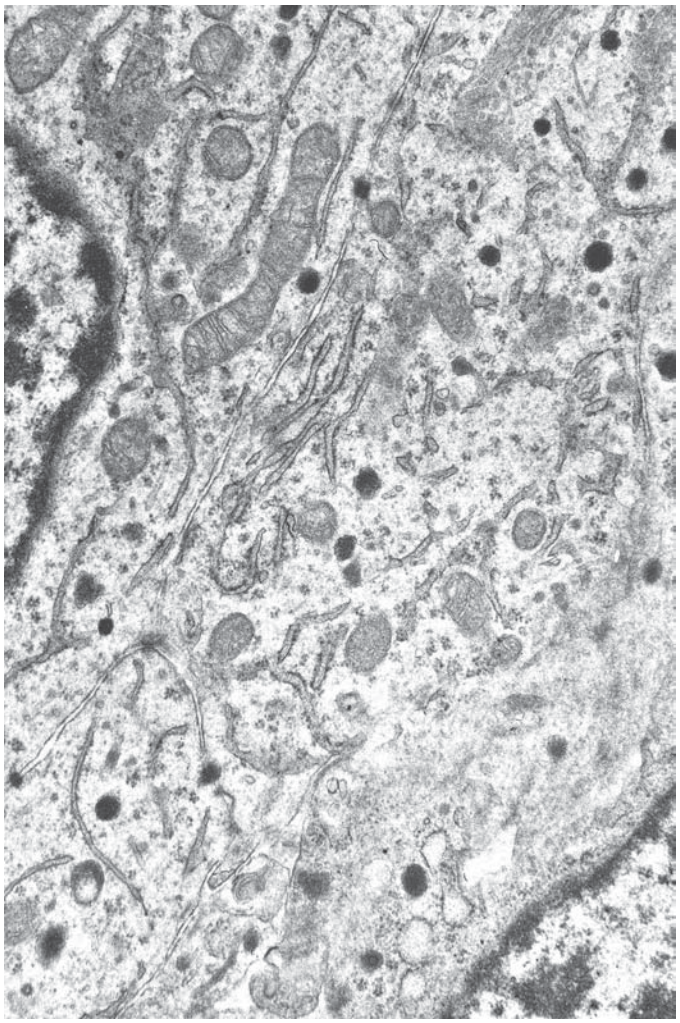

Figure 20 Ultrastructural aspect of a pancreatic VIPoma. Tumor cells are sparsely granulated and secretory granules are small and thin-haloed with a moderately dense core.

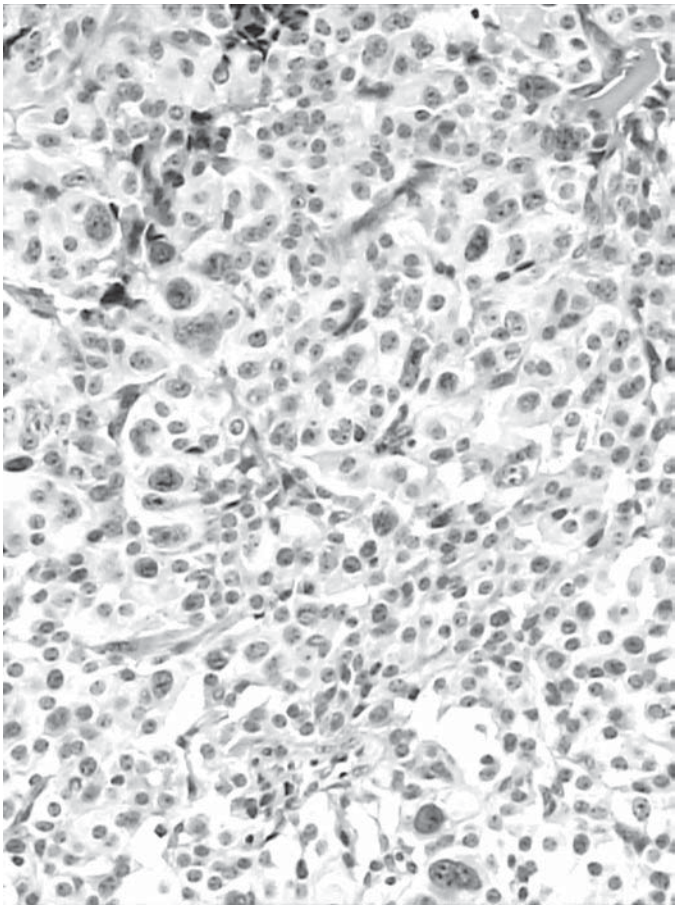

Figure 21 Malignant pancreatic gastrinoma composed of moderately atypical well differentiated endocrine cells forming solid structures.

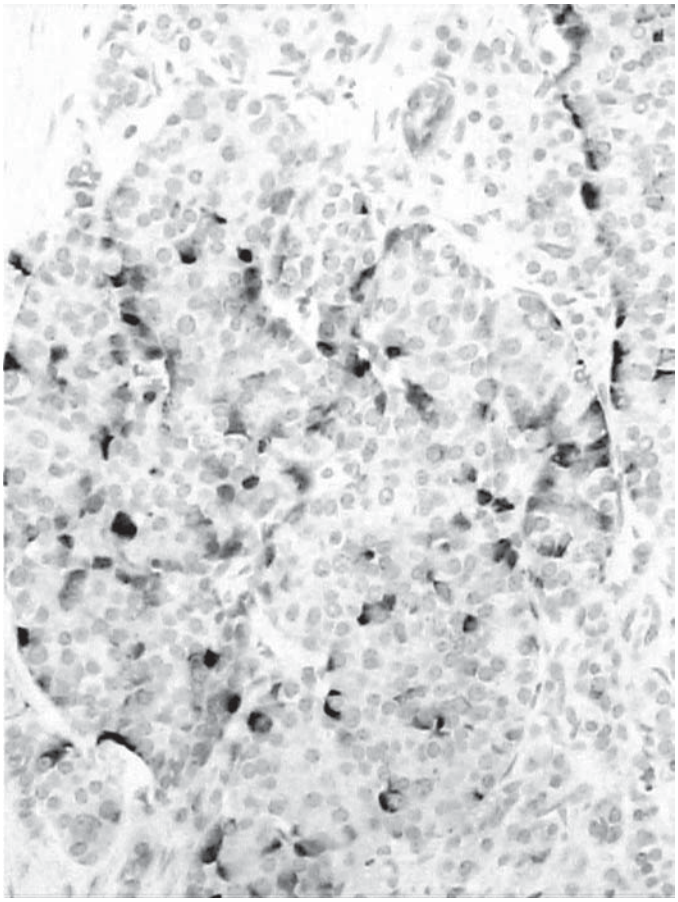

Figure 22 Gastrin immunoreactivity in a pancreatic gastrinoma.

Enterochromaffin (EC)-Cell Tumors Serotonin-producing (EC)-cell tumors of the pancreas associated with the classical carcinoid syndrome are very rare, accounting for about 45 cases described in the world literature, and predominantly malignant (well-differentiated endocrine carcinomas) [220,306]. Small, well-differentiated, EC-cell tumors that are nonmetastatic and lacking association with the carcinoid syndrome (with benign or uncertain behavior) have been also reported [220, 230]. Histologically, the tumor is more frequently formed by solid nests and/or trabeculae (Fig. 23), although a diffuse pattern with or without necrosis has been observed in large malignant tumors. Some tumors display a poorly differentiated aspect [307–309]. Well-differentiated tumors (mostly nonfunctioning EC cell tumors) are composed of cells with abundant serotonin-storing pleomorphic secretory granules (Fig. 24) while less-differentiated neoplasms have cells containing few secretory granules with low serotonin content.

Tumors Producing Acromegaly, Cushing's Disease, or Hypercalcemia These very rare tumor types are predominantly malignant, generally associated with poor patient outcome and with a high propensity for multiple hormone expression.

GRF-Secreting Tumors Growth hormone releasing factor (GRF) is a hypothalamic regulatory peptide that stimulates the release of GH from pituitary GH cells. Up to 17% of gastroenteropancreatic endocrine tumors have been found to express immunohistochemically GRF, and such reactivity has been detected more frequently in pancreatic than in gastrointestinal tumors [310–312]. Very few pancreatic GRF-secreting neoplasms causing acromegaly through GH hypersecretion from hyperplastic pituitary somatotrophs induced by GRF excess have been reported so far [313–318]. Histologically, these tumors show a trabecular, whorl-like meningotheliomatous, or even a paragangliod "zellballen" structure [220]. They are large tumors, often associated with proven liver or lymph node metastases, arising in relatively young subjects (median age of 34 yr). Some cases are associated with the MEN 1 syndrome [316] or with the Zollinger–Ellison syndrome with concomitant hypersecretion of GRF and gastrin [317]. A single case showing a pituitary metastasis has been reported [313]. In addition to GRF-secreting tumors, a case of GH-producing carcinoma of the pancreas associated with acromegaly has been reported [319].

ACTH-Secreting Tumors Pancreatic ACTH-secreting tumors are responsible for approx 10% of ectopic Cushing's-syndrome cases [220,320–325] and occur most frequently in adults with a prevalence among women, although a few cases have been described in children [324]. In addition, cases associated with the Zollinger–Ellison syndrome have been reported [325]. Tumors are generally firm, 2–12 cm in size, and distributed randomly in the pancreas. About 90% of these tumors metastasize to lymph nodes, liver, kidney, thyroid, peritoneum,

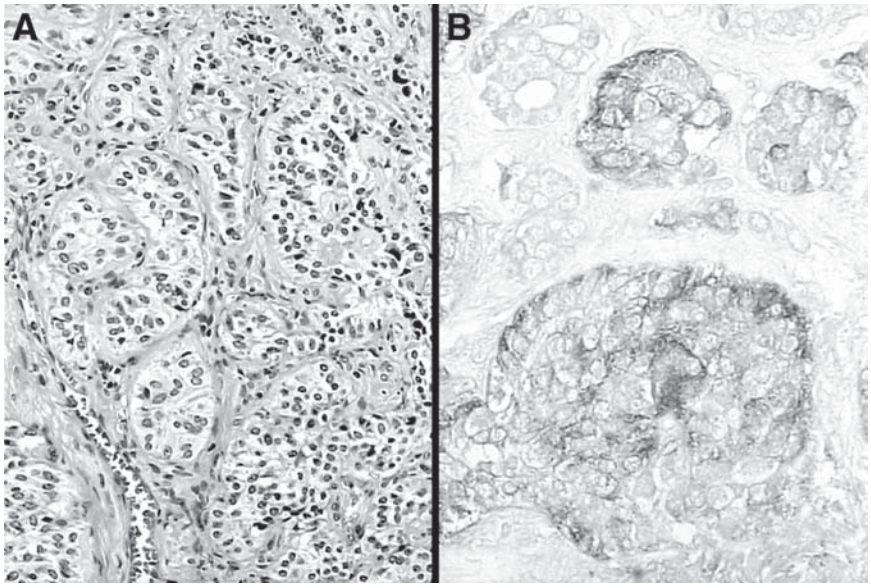

Figure 23 EC-cell tumor of the pancreas composed of solid nests of well differentiated cells (**A**), which are positive for serotonin (**B**).

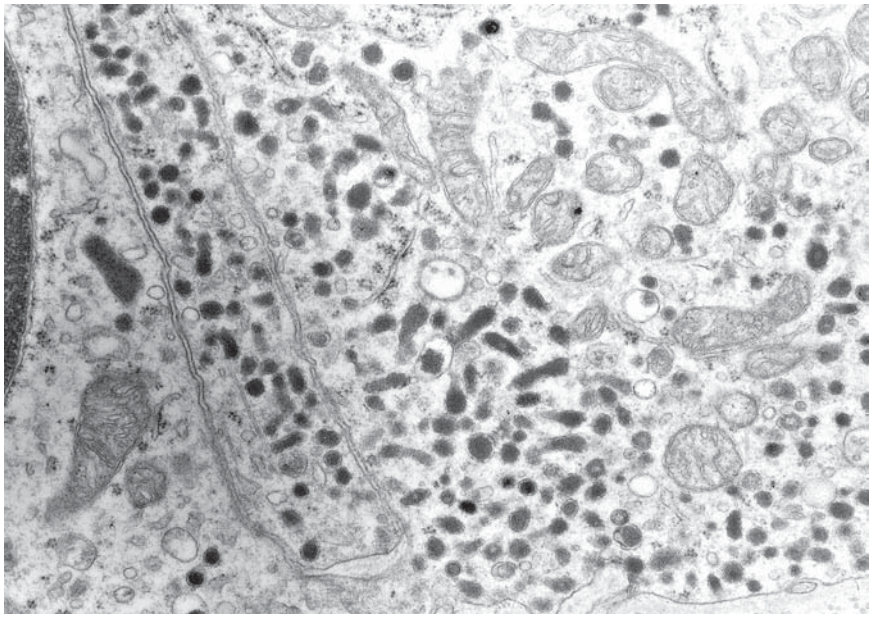

Figure 24 Ultrastructural features of a well differentiated EC-cell tumor. Tumor cells contain abundant pleomorphic secretory granules.

and bone and display an aggressive biological behavior [320]. Tumors are composed of small to medium-sized, moderately atypical cells, arranged in broad trabeculae, acini, or as solid growths separated by abundant fibrous stroma. In addition to these well-differentiated neoplasms, cases of small cell (poorly differentiated endocrine) carcinomas producing ACTH have also been reported [326].

Parathyroid Hormone-Secreting Tumors A few pancreatic endocrine carcinomas causing hypercalcemia and hyperparathyroid-type syndrome have been observed [220]. In very few cases there was an evident production of parathyroid hormone (PTH) [327–332], whereas in other cases lacking evidence of PTH secretion and involvement of PTH-related peptide (PTHrP) (parathirin) has been suggested [333]. Interestingly, PTHrP is expressed in the normal islet cells and is commonly detected in PETs [334]. However, elevations of PTHrP alone in the serum are not sufficient to induce hypercalcemia, and additional tumor-derived factors are needed to cause hypercalcemia [330].

WELL-DIFFERENTIATED NONFUNCTIONING ENDOCRINE TUMORS By definition, nonfunctioning endocrine tumors (NFETs) are neoplasms with endocrine differentiation in the absence of an evident clinical endocrine syndrome. However, when appropriately investigated by immunohistochemistry and electron microscopy, they show hormone-producing cells [223,258]. Among NFETs those causing symptoms due to the local growth or metastatic spread must be differentiated from incidentally detected clinically silent tumors because the former

are mostly malignant (Table 7). The apparent inactivity at the clinical level of NFETs is not clear and it has been tentatively explained by (1) insufficient hormone production (i.e., small neoplasms); (2) insufficient hormone release (i.e., inhibition of hormone release by somatostatin secreted by adjacent tumor cells); (3) regulated, rather than completely autonomous, hormone secretion, which renders hormone hypersecretion less prominent at the clinical level; (4) production of a relatively inert hormone having little impact on clinical symptomatology (i.e., pancreatic polypeptide, calcitonin, neurotensin); (5) synthesis and release of inactive molecular species of the entire hormone (i.e., proglucagon instead of active glucagon-29); and (6) insufficient clinical investigations [219].

NFETs have been detected in 0.3–1.6% of unselected autopsies in which only a few pancreatic sections were examined and in up 10% of autopsies in which the whole pancreas was systematically investigated both grossly and microscopically. NFETs represent approx 15–40% of surgically resected PETs [219, 246,335–337]. Interestingly, NFETs from autopsy series are generally small, benign neoplasms, composed mainly of well-granulated α and PP cells [338,339], while the majority of neoplasms from surgical series are malignant and associated with symptoms of an expanding mass [340,341]. In addition, multiple and small (mainly microadenomas) NFETs are characteristically found in pancreases of MEN 1 patients and are composed mainly of β, α, and PP cells [220,255]. No sex predilection has been observed in either the autopsy or the surgical series.

Grossly, the tumors have different features, varying from small, benign, capsulated nodules to large (with a maximum of 20 cm) locally invasive malignant neoplasms. In surgical series, clinically symptomatic NFETs arise more frequently in the head of the pancreas and have a size larger than 5 cm in 72% of cases [342].

Histologically, NFETs show the same architectural patterns observed in functioning neoplasms. Generally, well differentiated neoplasms with benign behavior display a trabecular–gyriform pattern, while well-differentiated endocrine carcinomas usually have a moderately defined, solid to broadly trabecular pattern. The cells are generally monomorph, of small to medium size, with no or mild nuclear atypia in the case of NFETs with benign or uncertain behavior, while malignant NFETs display a more prominent cellular atypia, more than two mitoses per 10 HPF and detectable angio/neuroinvasion.

Immunohistochemistry reveals positivity for general endocrine markers such as NSE, synaptophysin, PGP 9.5, and chromogranins. Despite the absence of endocrine symptoms, several neoplasms present cells positive for hormone peptides and among them the most frequently detected are PP, glucagon, somatostatin, serotonin, calcitonin, and neurotensin, in decreasing order [220]. Insulin, when present, is positive in only a few scattered cells while gastrin or VIP immunoreactivities have not been observed [230]. Small incidental nonfunctioning WDETs have a favorable prognosis. On the contrary, locally symptomatic large nonfunctioning WDECs are usually of low-grade malignancy with a mean survival of the patients ranging from 23 mo to 4.3 yr [219,343].

Calcitonin-Secreting Tumors Although scattered calcitonin-immunoreactive cells may be observed in some pancreatic endocrine tumors, such as somatostatin- or VIP-secreting neoplasms, pure calcitonin-secreting tumors are very rare in the pancreas. In our series of 63 NFETs of the pancreas, calcitonin-secreting tumors represent 12.6% of cases [344]. Patients are generally adults in the fifth decade of life with a slight female predominance. The patients do not show a characteristic endocrine syndrome, although they frequently suffer diarrhea and abdominal pain, which disappear after surgical resection [345].

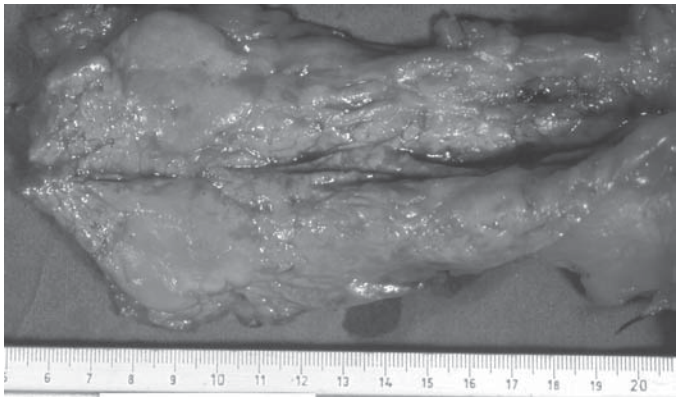

Figure 25 Gross appearance of a malignant calcitonin-secreting tumor of the body of the pancreas measuring about 3 cm. Although the tumor was confined to the pancreas, metastases to regional lymph node were found. (Color illustration appears in insert following p. 148.)

Calcitonin cell tumors are generally large (Fig. 25) (average diameter of 6.35 cm, range 2–20 cm), single, and equally distributed in the head, body, and the tail of the pancreas (Table 12). An association with the MEN 1 syndrome has been described in some cases [345]. In the majority of cases the tumors are malignant, showing metastases at the time of the diagnosis. Liver and regional lymph nodes are the more frequent sites of metastases, although brain and bone involvement has also been observed [345].

Histologically, the tumors show most frequently a trabecular pattern of growth although in some cases diffuse sheets of cells with focal pseudoglandular structures may be observed (Fig. 26). Unlike medullary thyroid carcinomas, amyloid deposits have not been observed in pancreatic calcitonin-secreting tumors [344]. Signs of local aggressiveness such as vascular and perineural invasion are frequently found, as well as foci of necrosis.

Tumor cells show immunoreactivity for general endocrine markers, calcitonin (Fig. 26), and, in addition, for other hormones such as PP, α-human chorionic gonadotropin (α-hCG), somatostatin, and neurotensin, in a minority of cells [344,345]. Follow-up data obtained from two series collecting a total of 14 cases are reported in Table 13.

Pancreatic Polypeptide-Secreting (PP)-Cell Tumors Although scattered PP-immunoreactive cells can be found in several pancreatic endocrine tumors [272,346] and approx 50% of patients with endocrine tumors of the pancreas have an elevated plasma level of PP [347], endocrine tumors composed mainly of PP cells are rare [220,272]. No distinctive endocrine syndrome correlated with PP hypersecretion has been identified so far and PP cell tumors, including large tumors producing high PP serum levels, present clinically as NFETs. The inci-

Table 12
Clinicopathological Features of Calcitonin-Secreting Tumors of the Pancreas

Sex	*Age*		*Ø (cm)*		*Site*			*Vasc.*	*Neur.*		
F/M	*Mean*	*(range)*	*Mean*	*(range)*	*H*	*T*	*met*	*inv.*	*inv.*	*Necrosis*	*References*
2/4	52	(30–74)	5.3	(2.5–10)	3	3	5/6				345
6/2	46	(30–61)	7.4	(2–20)	4	4	6/8	7/8	5/6	2/8	344

Ø, Diameter; H, head; T, tail; met, metastases; Vasc. inv., vascular invasion; Neur inv., perineural invasion.

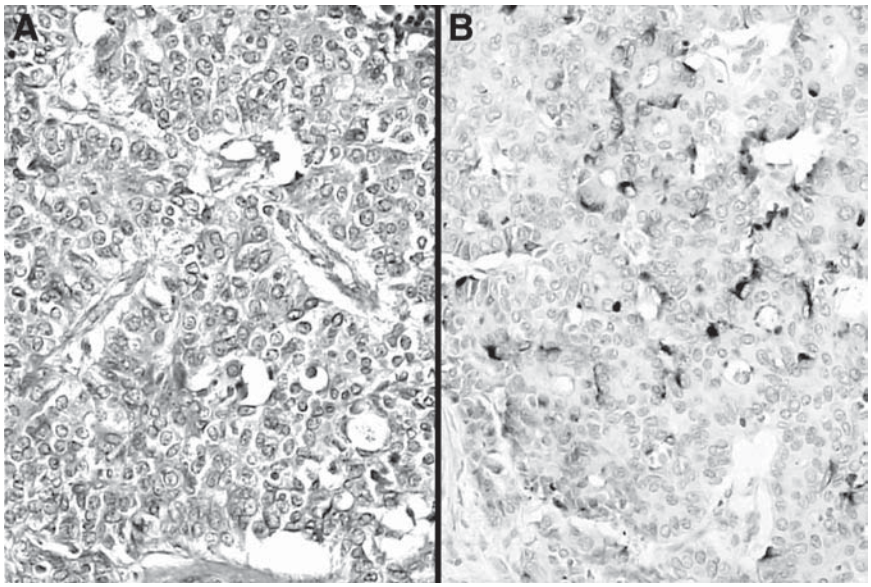

Figure 26 Microscopic features of the tumor shown in Fig. 25. Tumor cells are well differentiated and form solid-trabecular structures (**A**) and are strongly positive for calcitonin (**B**).

Table 13
Follow-up Data of Patients with Calcitonin-Secreting Pancreatic Tumors

6 AFD	18, 18, 24, 36, 77, 112 mo, respectively
2 AWD	36, 96 mo, respectively
3 DOD	2, 10, 24 mo, respectively
2 L	
1 DOC	

From refs. [344,345].
AFD, Alive free of disease; AWD, alive with disease; DOD, died of disease; L, lost at follow-up; DOC, died of other causes.

dence of this rare tumor type is difficult to assess but it has been evaluated to be about one or two per 10^6 population/yr [270].

Among PP cell tumors, small (<2 cm) (Fig. 27), clinically silent, densely granulated tumors with trabecular architecture are mostly benign. Malignant tumors are usually large (mean diameter: 8.1 cm, range 4–15 cm) and histologically they are solid and poorly granulated [348]. Tumors may occur in any part of the pancreas but they are more frequently found in the head.

Histologically, the prevalent architectural pattern is the trabecular one (Fig. 28). Tumor cells show mild nuclear atypia and rare mitotic figures. Neoplastic PP cells are variably stained with Grimelius' argyrophilic stain. The diagnosis of this tumor is based on the immunohistochemical positivity for PP of at least 50% of tumor cells.

POORLY DIFFERENTIATED ENDOCRINE CARCINOMAS

Poorly differentiated endocrine carcinomas (PDECs) are highly malignant neoplasms composed of small to intermediate and sometime large cells showing endocrine features. PDECs make up about 1–5% of all pancreatic malignant tumors [349] and about 2–3% of all pancreatic endocrine tumors of surgical series [220]. They occur in adults, predominantly in men aged between 40 and 75 [350,351]. In the majority of cases PDECs are nonfunctioning. The paraneoplastic syndromes, which are relatively common in patients with PDECs of other sites such as the lung, are seldom encountered in association with pancreatic neoplasms.

Grossly, PDECs are usually large (mean diameter of 4.2 cm) and are poorly demarcated, showing a gray-white color and areas of necrosis and hemorrhage. The tumors are more often located in the head of the pancreas where they invade adjacent organs. Metastatic spread to the liver, regional lymph nodes, and also extraabdominal sites is found in practically all cases [220,350].

Histologically, PDECs show features similar to those of small–intermediate cell carcinoma of the lung. Small to medium-

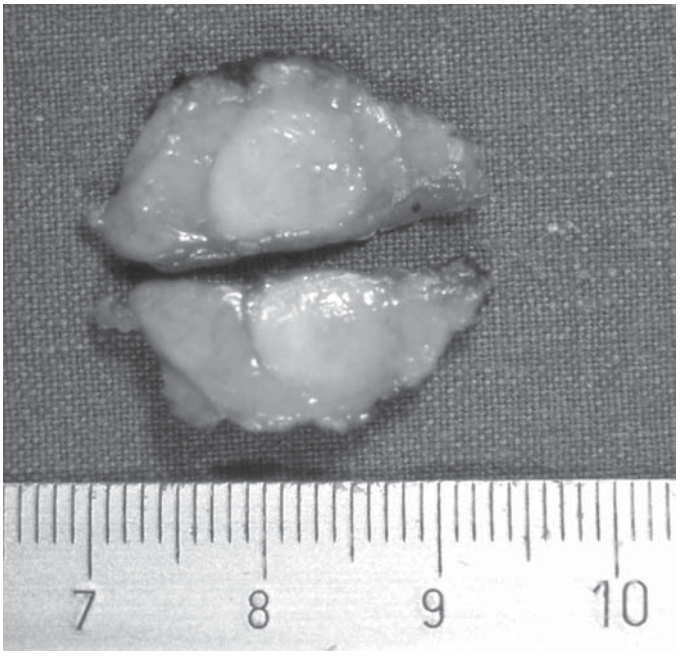

Figure 27 Nonfunctioning PP-cell tumor of the pancreatic head, incidentally found at surgery and enucleated. The tumor is well circumscribed and is about 0.5 cm in size. (Color illustration appears in insert following p. 148.)

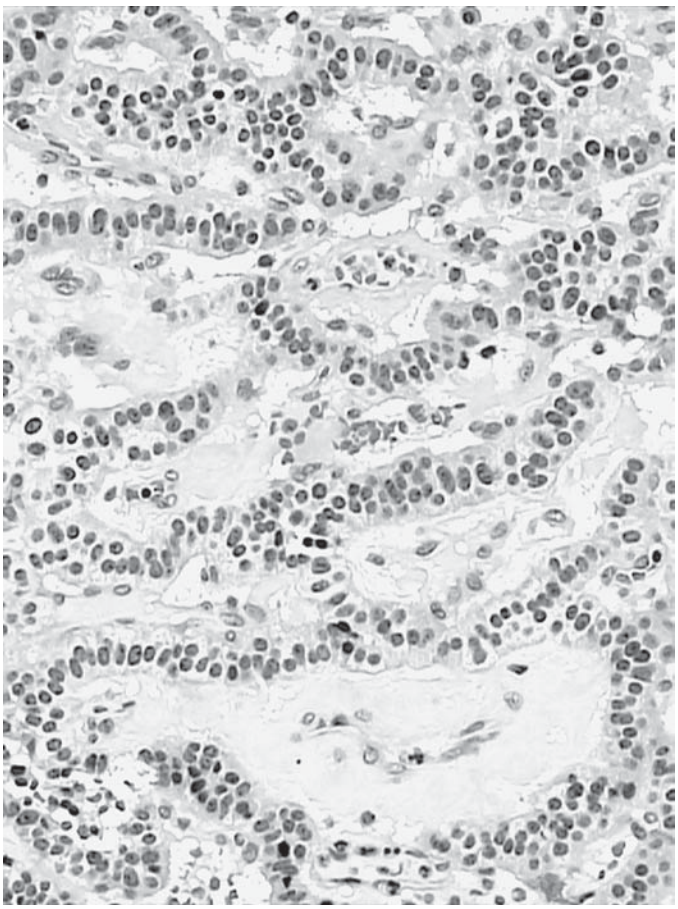

Figure 28 PP-cell tumor formed by trabecular–gyriform structures composed of well differentiated cells without significant nuclear atypia.

sized cells with markedly hyperchromatic round to oval nuclei, inconspicuous nucleoli, and poorly defined cytoplasmic borders form solid sheets and nests in which foci or large areas of necrosis are frequently observed (Fig. 29). The mitotic index (Fig. 29) is high as well as the Ki-67 proliferative rate [230]. PDECs are generally positive for NSE and synaptophysin whereas granular markers such as chromogranins are poorly expressed. Hormone peptide immunohistochemistry is generally negative although ACTH, calcitonin, and somatostatin have been occasionally detected [230,350,351]. Unlike well differentiated endocrine tumors, PDECs show diffuse and intense p53 oncoprotein nuclear accumulation [230].

At the ultrastructural level, tumor cells contain small amounts of granular endoplasmic reticulum, fairly abundant scattered ribosomes, bundles of intermediate filaments, and rare membrane-bound electron-dense secretory granules measuring 100–200 nm in diameter.

PDECs must be distinguished from metastases of small cell carcinomas of other sites, including lung, stomach, and colon. An important criterion for the differential diagnosis relies on the exclusion of a primary tumor outside the pancreas, considering that there are not site-specific immunohistochemical markers, including thyroid transcription factor 1 (TTF1), for distinguishing pancreatic from lung PDECs [352]. Differential diagnosis between PDECs and non-Hodgkin's lymphomas is based on the positivity in the latter for lymphoid markers such as CD45, CD20, or CD45RO.

MIXED EXOCRINE–ENDOCRINE TUMORS A mixed exocrine–endocrine tumor of the pancreas is defined as an epithelial neoplasm with a prevalent exocrine growth pattern and an endocrine component, representing at least one third of the tumor cell population [353]. The exocrine component may be benign (microcystic adenoma) [354,355] or, more frequently, malignant with the histologic features of either ductal adenocarcinoma or acinar carcinoma [356–358]. The biological behavior of the mixed exocrine–endocrine tumors depends on the exocrine component, when it is malignant. Immunohistochemistry is needed to confirm the mixture of the two components. The endocrine component stains positively for general endocrine markers (chromogranins and/or synaptophysin) and in several cases for pancreatic hormones, whereas the exocrine component is negative for hormones and endocrine markers, but is positive for carcinoembryonic antigen (CEA) and CA19.9 and in the case of mixed acinar/endocrine carcinomas for trypsin, lipase, and amylase. Electron microscopy may also be useful in identifying the endocrine and exocrine features of the tumors.

MOLECULAR GENETIC ALTERATIONS IN HUMAN PANCREATIC ENDOCRINE TUMORS Although several studies have been carried out in the last 10 yr, our knowledge about the pathogenesis of PETs is incomplete. Except for tumors developing in patients with specific genomic gene alterations, such as MEN 1 syndrome and von Hippel–Lindau (VHL) disease, the molecular events determining the uncontrolled growth of neuroendocrine cells are still unclear. In the following subsection, knowledge regarding genetic alterations as well as alterations in growth factors and growth factor receptors expression are reviewed.

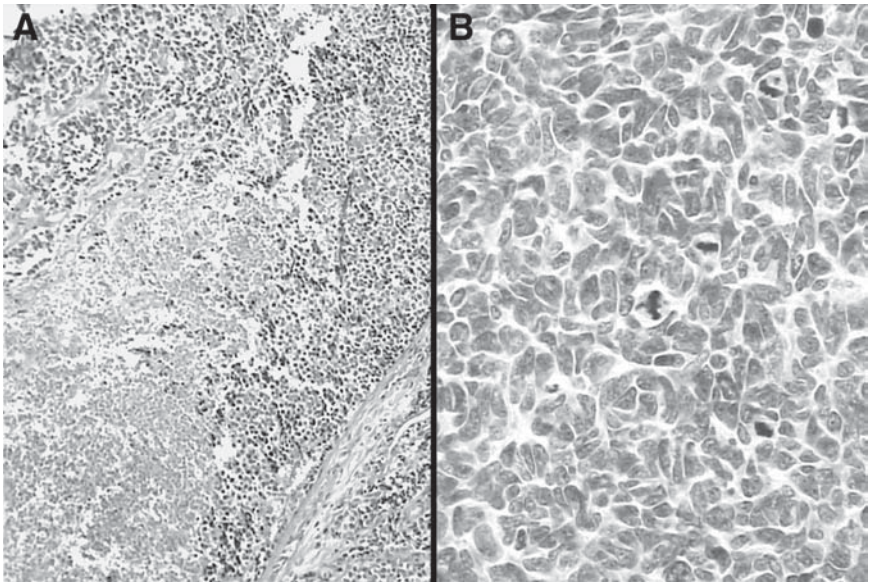

Figure 29 Poorly differentiated endocrine carcinoma of the pancreas. Low magnification (**A**) shows solid sheets of cells with abundant central necrosis. At higher magnification (**B**) cells show severe nuclear atypia and high mitotic rate.

Hereditary Forms of Pancreatic Endocrine Tumors A small percentage of PETs occur in patients with inherited syndromes, such as the multiple endocrine neoplasia type 1 (MEN 1) syndrome and VHL disease.

MEN 1 Syndrome MEN 1 (OMIM 131100) is a rare autosomal dominant disorder characterized by primary endocrine abnormalities involving the pituitary, parathyroid, endocrine pancreas, and duodenum. Hereditary pancreatic endocrine tumors occur in 80–100% of MEN 1 affected patients [359,360]. Most of these tumors are small, nonfunctioning, multiple, and usually benign (Fig. 30). Considering functioning tumors, 54% of affected MEN 1 patients develop gastrinomas, 21% develop insulinomas, and fewer than 5% develop the other functioning PETs [360]. MEN 1-associated PETs have an earlier age at onset and a much higher rate of postoperative recurrences as compared with their sporadic counterparts. They represent the most common cause of death in patients with MEN 1 [360]. The basis of familial MEN 1 inheritance is a germline mutation in the *MEN1* tumor suppressor gene recently localized to a small genomic interval at 11q13 by LOH studies and finally identified by positional cloning [361–363]. All MEN 1 families reported so far have tight linkage to 11q13 locus and mutation analysis revealed that the *MEN1* gene is frequently altered in MEN 1 families. Heterozygous germline mutations scattered throughout the MEN 1 protein coding region have been identified in 95% of probands/families with patients sharing at least three major lesions of the syndrome and first-degree relatives affected by one (or more) MEN 1-related lesions. Of the more than 300 unique mutations reported, >70% of these are truncating mutations resulting from frame shift (deletions, insertions, deletion/insertions or splice-site defects) and nonsense mutations [364,365]. Of the *MEN1* missense mutations reported in the literature >95% occurred at residues highly conserved among human, mouse, zebrafish, and *Drosophila* [365–369], suggesting thereby a functional/pathogenic significance. No genotype–phenotype correlations have been established to date between the type and location of *MEN1* mutations and the clinical features of MEN 1 in probands and families [370–375]. Most families

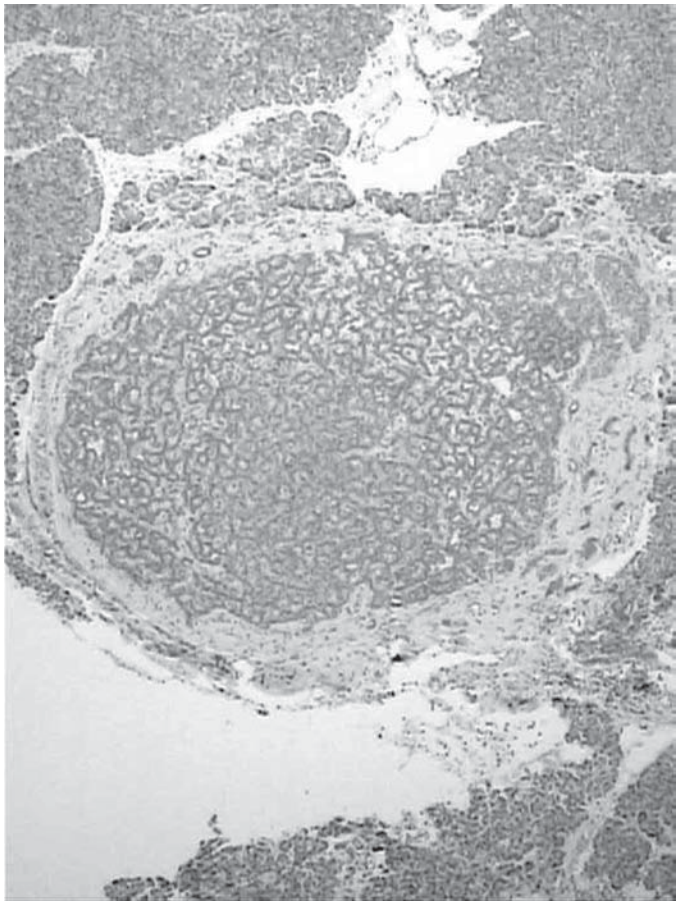

Figure 30 Characteristic pancreatic microadenoma in a patient with MEN 1 syndrome.

without demonstrable *MEN1* mutations display an atypical clinical pattern, that might suggest genetic heterogeneity of the disease or the occurrence of phenocopies with lesions that are commonly observed in the non-MEN 1 individuals, such as primary hyperparathyroidism and prolactinoma [376,377]. Lastly, when MEN 1 is strongly suspected, mutations might occur in an unknown part of the *MEN1* sequence, such as the 5' regulation region for which functional characterization is in progress [377, 378]. *MEN1*-associated tumors show somatic alteration of the remaining wild-type allele by chromosome loss, chromosome loss with duplication, mitotic recombination, or another localized event such as point mutation. These data strongly suggest a molecular mechanism of endocrine tumorigenesis by the inactivation of menin, the protein product of the *MEN1* gene. Although the molecular mechanism for the growth suppression of menin and its physiological role are not known, there is increasing evidence that menin may function in DNA repair or synthesis and that the molecular mechanism for its growth suppression may be mediated through its interactions with transcription factors [379–381]. Peripheral blood leukocytes and fibroblasts from MEN 1 patients treated with diepoxibutane revealed an increased frequency of spontaneous chromosomal alterations and of mitoses with premature centromere divisions [382–384]. These data derived from in vitro studies are in agreement with recent findings reported by Hessman et al. [385] in a genome-wide LOH screening of 23 MEN 1 pancreatic lesions. In this study the authors observed multiple allelic deletions involving chromosomes 11, 6, 8, 10, 18, and 21 and a high level of inter- and intratumor heterogeneity, suggesting the presence of chromosomal instability. Overall such findings confirm the relevance of the *MEN1* gene for pancreatic endocrine tumorigenesis and highlight the need for a functional assay for menin activity supporting the available methods for *MEN1* germline mutation testing (including dideoxyfingerprinting, heteroduplex analysis, single-strand conformation polymorphism, or direct sequencing of selected regions) not only to make a highly confident genetic diagnosis but also to design adequate treatment modalities for *MEN1* tumors.

von Hippel–Lindau Disease VHL syndrome (OMIM 193300) is a dominantly inherited cancer syndrome predisposing to a variety of malignant and benign neoplasms, most frequently retinal, cerebellar and spinal hemangioblastoma, renal cell carcinoma, pheochromocytoma, and cystic and/or endocrine pancreatic tumors [386]. VHL-related pancreatic tumors are mostly exocrine microcystic adenomas but 10–15% of patients with VHL could be affected by endocrine tumors [387–389]. VHL-related PETs have typical morphologic characteristics, consisting of solid, trabecular, or glandular structures composed in about 60% of cases of clear cells with vacuolated lipid-rich cytoplasm [390,391] (Figs. 31 and 32). Most tumors are multiple and nonfunctioning and 30–40% of them demonstrate focal positivity for pancreatic polypeptide, somatostatin, glucagon, and/or insulin [390]. Like VHL-associated renal tumors and retinal and/or cerebellar neoplasms, pancreatic islet cell tumors are markedly vascular. Most of these tumors are slow growing and asymptomatic, but some cases can grow rapidly or metastasize. Despite the variety of tumor types observed clinically in this disorder, progression to malignancy in VHL disease is associated primarily with the development of renal carcinomas (RCC) and pancreatic islet cell tumors [390,391]. VHL-related PETs might be distinguished from *MEN 1*-related tumors based on (1) the absence of primitive duodenal tumors; (2) frequent nonfunctioning lesions with focal positivity for pancreatic polypeptide, somatostatin, glucagon, and insulin; (3) a clear-cell

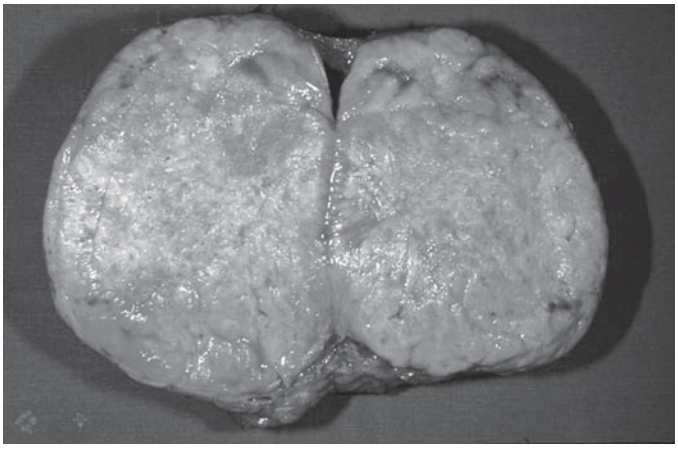

Figure 31 Macroscopic view of a pancreatic endocrine tumor presented in a patient with the von Hippel–Lindau disease. The tumor is well circumscribed, encapsulated, and mostly yellow. (Color illustration appears in insert following p. 148.)

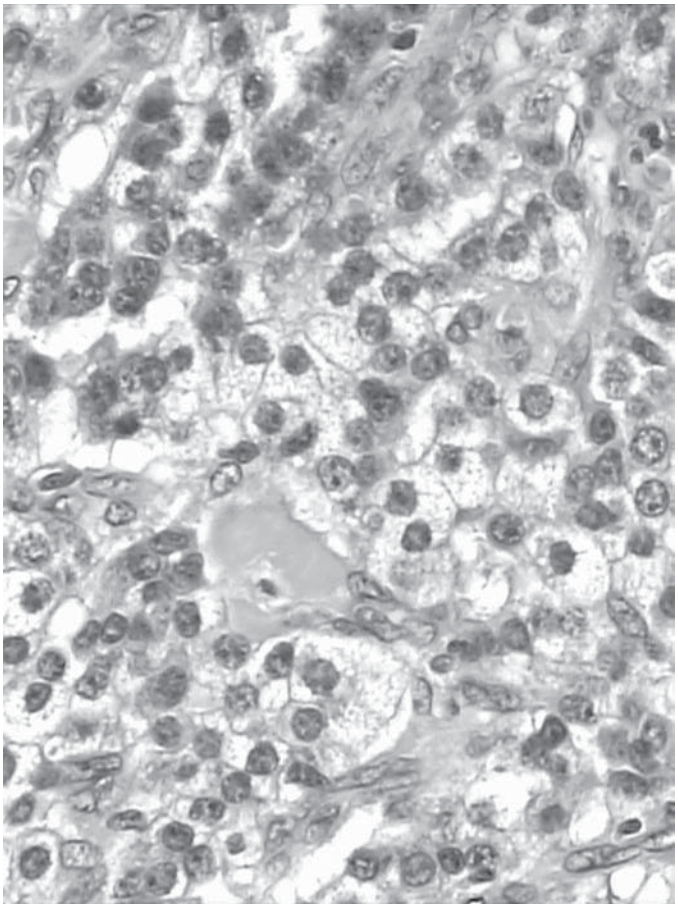

Figure 32 Microscopic appearance of the tumor shown in Fig. 31. Tumor cells from the yellow areas show a characteristic clear vacuolated cytoplasm.

morphology related to intracytoplasmic lipid and myelin accumulation; and (4) frequent occurrence of microcystic adenomas around the clear-cell tumors [391]. The *VHL* gene maps to chromosome 3p25 and has been shown to be a tumor suppressor gene with multiple functions including regulation of angiogenesis, ubiquitination, as well as a gatekeeper function in the G_0/G_1 checkpoint [392–396]. Zbar et al. [397] identified germline mutations in 300 out of 469 (63%) VHL families from North America, Europe, and Japan and genotype–phenotype correlations have been established between the type and location of *VHL* gene mutations and distinct cancer phenotype. The catalog of *VHL* germline mutations with phenotype information provided by Zbar et al. [397] should be useful for diagnostic and prognostic studies of VHL and for studies of genotype–phenotype correlation in this disorder. *VHL*-associated tumors show somatic alteration of the remaining wild-type allele by allelic loss or, more rarely, by hypermethylation of the normally unmethylated CpG island in the 5' region of the gene [398]. In addition, it was recently suggested that loss of heterozygosity of genetic loci distinct from and mapping proximal to the *VHL* gene may be correlated to malignant conversion in *VHL*-associated tumorigenesis [391]. *VHL* gene sequencing has been useful in presymptomatic diagnosis of VHL disease and in some clinical states suggesting differential diagnosis with MEN 1 and MEN 2 [399].

Sporadic Pancreatic Endocrine Tumors The vast majority of PETs occur sporadically and, although detailed information is now available on the phenotypic and functional profile of these neoplasms, there is very little information on the genotype of well-differentiated tumors and virtually no information for poorly differentiated forms. Furthermore, currently used immunohistochemical and molecular markers are of limited value in predicting the malignancy of PETs.

Somatic mutations of the *MEN1* gene have been found in roughly 30% of sporadic PETs [400–402]. The frequency of *MEN1* mutation differs among the tumor types: alterations in *MEN1* have been found in 54% (15/28) of gastrinomas, 50% (4/8) of VIPomas, 2/3 of glucagonomas, and 1/1 somatostatinoma, but in only 7% (4/54) of insulinomas [401,403–406]. Among nonfunctioning tumors the reported mutation frequency is variable [403,406–408], summing up to 26% in the largest series investigated (31 cases). A genotype–phenotype correlation has been established among sporadic gastrinomas. Primary pancreatic or so-called "lymph node" sporadic gastrinomas exhibit mutations in exon 2 of the *MEN1* gene much more frequently than duodenal gastrinomas. Small (<1 cm) primary tumors are also less likely to have a mutation involving exon 2 of *MEN1*. No genotype–phenotype correlation was observed with regard to postoperative disease-free status and overall patient outcome. Allelic deletion on 11q is frequently found in sporadic PETs [403,406,409] and combining data from all studies referred, it appears that the LOH rate is usually two to three times higher than the frequency of mutations of the *MEN1* gene. This suggests that LOH at 11q13 *per se* is not an indicator of *MEN1* mutations. Such findings can be explained by other mechanisms of *MEN1* gene inactivation, such as methylation of the promoter or the presence of mutations in unexamined noncoding regions. It also seems likely that the higher frequency of allelic deletions is an indicator for the inactivation of another tumor suppressor gene on 11q.

To clarify the role of the *VHL* gene in the pathogenesis of sporadic pancreatic endocrine tumors, extensive mutational screening of the gene was recently performed [393,403,410, 411]. Overall the data reported indicate that the mutation of the *VHL* gene is not a frequent event in sporadic PETs but its role in endocrine tumorigenesis cannot be excluded because somatic inactivating mutations were found in a small proportion of NF-PETs [403]. Allelic deletion on 3p was frequently found in sporadic PETs and a positive correlation between 3p loss and malignant tumor behavior was observed by different authors [410,412]. Chung et al. [410] and Barghorn et al. [412] identified the smallest common region of allelic loss between 3p25.3–p25.1 and 3p23, suggesting the presence of another tumor suppressor gene centromeric to the *VHL* gene. Barghorn et al. [412] observed that most nonfunctioning tumors lost the entire chromosome 3 during progression to the metastatic phenotype whereas functioning PETs lost only parts of chromosome 3p and rarely the entire chromosome 3p. Because the pattern of allelic deletions at 3p differs among PET subtypes and microsatellite markers at 3p25.3–p23 may have already been lost at an early tumor stage, caution was suggested with respect to the use of 3p markers in distinguishing clinically benign from potentially malignant PETs.

The four genes frequently altered in common ductal adenocarcinomas, including K-*ras*, *p53*, *p16,* and *DPC4*, have also been examined in PETs. The K-*ras* and *p53* genes have no significant role in the pathogenesis of PETs [413,414]. Nevertheless, a high frequency of LOH on chromosome 17p has been described, mutations were found in two malignant cases, and hyperexpression/accumulation of the p53 protein was reported in malignant poorly differentiated cases [230,413–415].

Controversial data are reported for well-differentiated PETs about mutations of the *DPC4* gene. Mutations were described in 55% of nonfunctioning tumors as compared to none of 16 functioning cases [416]. On the contrary, in recent studies no mutations of the *DPC4* gene were detected in tumors with allelic deletions at 18q21 loci [417,418].

Muscarella et al. [419] reported the presence of *CDKN2A/p16* promoter hypermethylation or homozygous deletion in a limited number of pancreatic gastrinomas and nonfunctioning PETs (a total of 14 cases investigated). These data were confirmed by Lubomierski et al. [420], who reported loss of expression of at least one of the tumor suppressor genes *CDKN2A/p16*, *CDKN2B/p15*, and *CDKN2D/p14* localized as a gene cluster at 9p21. mRNA transcripts of these genes were lost most frequently in nonfunctioning PETs (57%) and less commonly in insulinomas (30%) and gastrinomas (22%). Such findings suggest that gene silencing by *de novo* methylation may be a crucial event in the endocrine tumorigenesis, as methylation errors, in association with a repressive chromatin structure, have been identified as critical determinants in the progression of different types of tumors [421].

Other oncosuppressor genes such as *RB1, PTEN, BRCA2* [422–424] and common oncogenes including *myc, fos,* c-*erbB-2,* and *sis* [425] have been examined in PETs but no genetic alterations were observed.

In recent years, to identify molecular markers of malignancy that could be crucial for the prognostic evaluation of sporadic PETs, comprehensive genome-wide approaches such as comparative genomic hybridization (CGH) and high-resolution allelotyping were chosen to analyze chromosomal and genetic imbalances during the progression of endocrine pancreatic tumors [409,426–428]. A wide spectrum of genetic aberrations was detected in PETs including chromosomal losses of 1p, 3p, 3q, 6q, 11q, Xp, Xq, and Y and gains of 5q, 7q, 9q, 14q, and 17q. Specific chromosome regions frequently reported as unbalanced by genome-wide approaches were analyzed by detailed mapping of allelic losses with the aim of correlating molecular data with clinical and histopathological parameters and thereby identifying new locations of tumor suppressor genes potentially involved in PET pathogenesis. LOH studies on PETs indicated different chromosomal regions such as 1p, 3p, Xp, and 6q, whose deletion is associated with more aggressive behavior [410,413,429,430]. Barghorn et al. [431] reported a LOH analysis on the long arm of chromosome 6 in a large series of sporadic PETs (109 tumors) demonstrating a high percentage of 6q losses (in 62% of cases examined) and narrowing down two common regions of allelic deletion at 6q22.1 and at 6q23–q24. The authors observed that these regions were significantly more often deleted in malignant than in benign PETs, indicating that these loci might harbor tumor suppressor genes critically involved in the malignant progression of PETs. Interestingly, losses of larger regions on 6q or the entire chromosome 6 were strongly associated with metastatic disease. Analogously, Pizzi et al. [430] suggested that tumor suppressor gene(s) mapping on the X chromosome may be involved in the malignant evolution of foregut but not of midgut or hindgut endocrine neoplasms and that extensive LOH at the X chromosome may represent a molecular marker of malignancy of these tumors.

An interesting finding from CGH studies regards the strong correlation between the total number of genomic changes per tumor and both disease stage and tumor size. Speel et al. [426] found more genetic alterations in PETs >2 cm in diameter than in PETs ≤ 2 cm in diameter, suggesting that genetic instability appears in the smaller tumors and that with the increase of tumor size additional genomic changes accumulate, resulting in malignant transformation. Intriguingly, a pattern of allelic imbalances involving focal and small chromosomal regions seems to be characteristic of functioning PETs compared with recurrent imbalances of entire chromosome arms observed in nonfunctioning PETs. On the other hand, in a recent high-resolution allelotyping analysis of only nonfunctioning PETs (32 cases examined), Rigaud et al. [409] underlined the existence of two different allelotypes among NF-PETs: one aneuploid or multiploid with a high degree of large chromosomal allelic deletions and the second, diploid, showing a small number of scattered losses with no apparent specific localization. In this study, survival analysis showed that no specific chromosomal alteration was associated with outcome, whereas ploidy status is an independent factor adding prognostic information to that given by the proliferative index evaluated with Ki-67 immunohistochemistry.

Overall the data referred to up to now appear to be too scanty and fragmentary to define with certainty specific molecular markers of malignancy useful for the prognostic evaluation of these neoplasms. Currently, the ploidy status and the evaluation of the global level of chromosomal instability of the tumors appear to be the most informative genetic factors with prognostic significance. Although limited by the relatively small and heterogeneous series of investigated cases, the scenario emerging from the aforementioned data depicts a complex mechanism of endocrine tumorigenesis often involving tumor-type restricted genetic defects. Overall these findings emphasize the need for further studies of the molecular pathogenesis of PETs, carefully subdivided according to their morphofunctional profile.

Growth Factors In recent years several studies have demonstrated that PETs express several growth factors and growth factor receptors. The role of these peptides in pancreatic endocrine tumor development is not clear yet. Generally, the results of these studies seem to indicate that *de novo* expression of growth factors and/or their receptors is involved in tumor development but not in tumor aggressiveness. Neither acidic (aFGF) nor basic (bFGF) fibroblast growth factor are expressed in PETs whereas their receptors (FGFR1-4) have been found immunohistochemically in the majority of tumors [84]. Interestingly, these receptors have a specific distribution in normal islet cell types (Table 2) but in PET types they are overexpressed even in cell types that in normal islets lack those particular FGFRs. TGF-α has been found in several PETs [432,433]. However, its specific receptor (EGFR) is expressed at a lower level and frequently only one of the two receptor domains is present, indicating the expression of an incomplete form of the molecule [433]. Hepatocyte growth factor (HGF) has been recently found to be immunohistochemically expressed in several PETs, including nonfunctioning neoplasms, gastrinomas, VIPomas, and insulinomas [434]. Some of these tumor types also express the HGF receptor, named met [434,435], suggesting the existence an autocrine/paracrine mechanism regulating tumor growth. HGF is known to be a stimulator of tumor spread but its expression is not correlated with metastatic rate in PETs [434]. Insulin-like growth factor, TGF-β, vascular endothelial growth factor, platelet derived growth factor, and their receptors have been also observed in a few PETs, but their role in PET biology remains to be clarified [113,435–438].

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17 Fine Needle Aspiration Cytology of Endocrine Glands

ANNA B. O'GRADY MD, KRISTEN A. ATKINS MD, AND CELESTE N. POWERS MD, PHD

INTRODUCTION

Although fine needle aspiration is considered a relatively recent diagnostic procedure, one of the earliest known reports of this technique was documented in 1847 by M. Kun, who noted that "in this manner a microscopic examination of the tumour can be practised on the living subject, and its nature ascertained before having recourse to an operation" [1]. Determining the nature of an unknown lesion without relying on surgical intervention is the primary indication for fine needle aspiration biopsy (FNAB), which has finally gained widespread acceptance as a diagnostic technique, after many years of lukewarm interest. Drs. Martin and Stewart briefly popularized the technique in the early 1930s at the Memorial Center for Cancer and Allied Diseases in New York [2,3], but it was not until the 1970s that FNAB was practiced with any frequency in the United States. This surge in popularity followed a wave of interest in the technique that began with a group of clinicians in Scandinavia and Europe, and that was accompanied by several improvements in the technique and seminal publications regarding the method [4,5]. By the early 1980s, FNAB had become reasonably well established in the United States [6,7]. Since that time, the technique has seen dramatic increases in use by clinicians, radiologists, and pathologists.

One of the first and most popular indications for this technique has been in patients with lesions of the thyroid gland [8–11]. Many experts believe that FNAB should be the first test ordered in the evaluation of a thyroid nodule [12]. Most cancers can be identified by aspiration cytology, and for those entities that still evade diagnosis, ancillary testing performed on the aspirate material is often helpful, and will continue to provide increasing amounts of information as molecular testing becomes more extensive. Parathyroid lesions, if palpable, are also amenable to FNAB and may provide useful information. An experienced interventional radiologist, via ultrasound guidance, can aspirate nonpalpable lesions of both the thyroid and the parathyroid. In addition, utilizing interventional radiology, lesions of the adrenals and the endocrine pancreas, as well as gastrointestinal endocrine lesions, are also accessible to FNAB, which frequently yields useful information in these types of lesions, again without the morbidity associated with open, or even laparoscopic, biopsy techniques.

The success of the FNAB technique depends largely on the experience and skill of the aspirator [13–15]. It is highly preferable for the same individual to take the history, examine the patient, perform the aspirate, and prepare and interpret the slides. FNAB of a palpable lesion is usually performed with a 25- or 23-gauge needle, with or without an attached 10- or 20-cc syringe and aspiration device, such as a syringe holder. After cleaning the skin with a disposable alcohol pad, the nondominant hand is used to isolate and steady the target lesion. The needle is inserted into the mass and, if a syringe is used, suction is applied. The needle is passed back and forth within the mass several times to dislodge tumor cells and to collect them within the barrel of the needle. The process continues just until cellular material becomes visible in the hub of the needle. Suction is released prior to withdrawing the needle.

The cellular material is expelled onto a glass slide and smeared with another glass slide. Smearing techniques vary, but all are intended to produce a thin layer of cells while preserving cellular morphology. Bloody aspirates or cyst fluid can dilute the cellular material, and special smearing techniques are sometimes used to remove the excess fluid from these specimens [10]. The prepared slides are either fixed in ethanol (for Papanicolaou staining) or air dried (for Diff-Quik® staining). Additional material may be collected in RPMI solution for ancillary testing and/or cell block preparation. Monolayer collection solutions are also used in some centers.

If possible, it is best to perform multiple aspirates of the same nodule in order to sample the lesion thoroughly and to ensure collection of a sufficient amount of material for any necessary ancillary studies. Rapid staining of selected slides using Diff-Quik®, hematoxylin & eosin (H&E), or modified Papanicolaou stains can be used to assess specimen adequacy and to allow triage of any additional material obtained. Some authors advocate the use of a local anesthetic to facilitate repeat aspirations, but this is typically not necessary for superficial, palpable lesions, as FNAB is a rapid and well-tolerated procedure.

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Table 1
The Spectrum of Follicular Lesions

Cytologic criteria	*Colloid nodule*	*Nodular goiter*	*Follicular neoplasm*
Cellularity	Low	Moderate	High
Follicular cells	Scant	Moderate	High
Colloid	Abundant	Moderate to abundant	Scant to absent
Pattern	"Cracked" colloid	Follicles, honeycomb sheets	Microfollicles, trabeculae, single cells
Follicular cells			
Nucleus	Bland	Bland	Enlarged, crowded, overlapping
Chromatin	Even	Even	Granular
Nucleolus	Indisinct	Indistinct	Distinct
Other cell types	Occ. histiocyte/macrophage	Occ. lymphocytes Hemosiderin-laden macrophages	None

THYROID

The primary indication for FNAB of the thyroid gland is the presence of a solitary palpable nodule, particularly if the nodule is classified as "cold" on a radionucleotide imaging scan, or if it is firm, irregular, fixed to the surrounding structures, or rapidly growing. Other indications include the presence of a dominant or growing nodule in a background of multinodular goiter; diffuse goiter when the differential diagnosis includes thyroiditis, hyperplasia, or lymphoma; palpable lesions in a patient with a family history of thyroid carcinoma; or lesions believed clinically inoperable. Relative contraindications include a recent history of anticoagulant therapy or the presence of a bleeding diathesis, due to the risk of hematoma formation. Suspicion of hyperthyroidism or thyrotoxicosis is also a contraindication, as it is possible to induce thyroid storm with the FNAB procedure in this setting. A family history of thyroid malignancy or a clinical history of ionizing radiation should increase the aspirator's suspicion of malignancy. The sensitivity and specificity of FNAB of the thyroid gland are both >90% [6,14,16].

Several authors have suggested objective adequacy criteria for the evaluation of thyroid aspirates [17–21]. The specific criteria vary with the author, but typically involve counting follicular cells and cellular groups. Under optimal circumstances, when an experienced pathologist–aspirator is able to examine the patient, perform the aspiration, and examine the aspiration material grossly, rigid adequacy criteria may not be necessary. When this type of involvement is not possible, it is helpful to establish and adhere to minimum requirements for adequacy, to decrease the incidence of false-negative diagnoses [22].

The spectrum of follicular lesions that present as nodules ranges from low cellularity colloid nodules to cellular follicular lesions and follicular neoplasms. Aspiration biopsy can be successfully used as a triage procedure for these lesions, and can also be used to assess diffuse inflammatory processes such as thyroiditis. In addition, FNAB is useful as a diagnostic procedure for malignancies including papillary, medullary, and anaplastic carcinomas.

NODULAR GOITER A dominant nodule within a multinodular thyroid is one of the most common presentations for aspiration biopsy. The majority of these nodules represent non-neoplastic lesions that yield variable amounts of colloid and follicular cells on aspiration. The ratio of follicular cells to colloid is key to distinguishing non-neoplastic nodules (goiter) from follicular neoplasms (Table 1). The greater the amount of colloid in relation to follicular cells, the less likely the nodule is neoplastic [9,10] (Fig. 1). Other features that help distinguish goiter from follicular neoplasms is the presence of multiple cell types, for example, follicular cells, Hürthle cells, lymphocytes, and macrophages. In addition, follicular cells in non-neoplastic lesions are often arranged in follicles, spherules, or evenly spaced as "honeycomb" sheets (Fig. 2A,B). Although their nuclear size may vary (benign endocrine atypia), they have bland nuclear morphology.

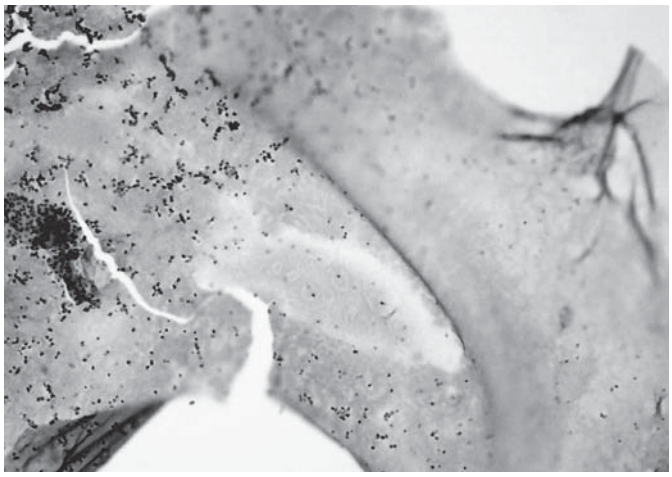


Figure 1 Colloid nodule. Colloid is the predominant feature in these aspirates, it often appears as a wavy sheet that can fracture or detach from the slide surface. Papanicolaou stain, original magnification ×200.

Cystic lesions can be particularly difficult to interpret, primarily due to low cellularity. Most of these lesions are not true cysts, but rather are areas of cystic degeneration in a background of nodular goiter. Because thyroid aspirations are frequently bloody or cystic, a syringe and holder may be particularly useful to collect any fluid that may be aspirated from the nodule. The features that indicate cystic change include degenerative/regressive changes of follicular cells, abundant hemosiderin-laden macrophages, and, in longstanding cases, dystrophic calcification and cholesterol crystals (Fig. 3). Although cystic degeneration commonly occurs in benign thyroid lesions,

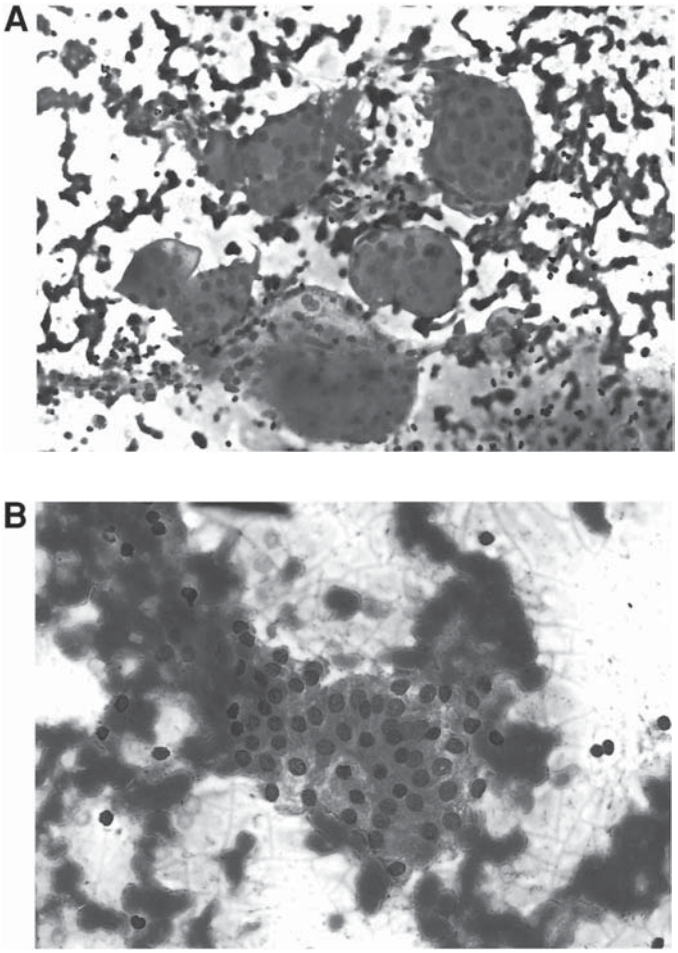


Figure 2 (**A**) Non-neoplastic thyroid. Thyroid follicles are often aspirated as intact three-dimensional spherules. Thyroid aspirates from benign lesions also typically contain moderate amounts of blood and colloid. Papanicolaou stain, original magnification ×200. (**B**) Non-neoplastic thyroid (goiter). Fragment of bland follicular cells arranged as a flat honeycomb sheet. Stripped follicular cell nuclei are also present. Cracked colloid and blood are background elements. Diff-Quik® stain, original magnification ×200.

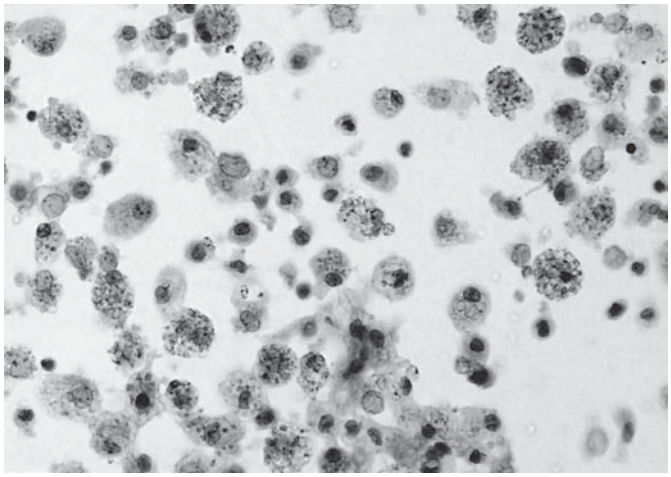


Figure 3 Cystic degeneration in non-neoplastic thyroid. Histiocytes and hemosiderin-laden macrophages are the predominant feature. Occasional degenerated follicular cells may be seen. Papanicolaou stain, original magnification ×200.

it can also occur in thyroid malignancies, particularly papillary thyroid carcinoma. When papillary carcinoma presents as a cystic lesion, aspiration often decompresses the cyst; however, fluid usually reaccumulates quickly, often in a matter of minutes or hours. The cyst fluid should be examined for atypia and features suggestive of papillary carcinoma. Often the fluid is paucicellular with scattered single cells resembling macrophages. Close scrutiny is necessary in these cases. The presence of even a single intranuclear inclusion in this setting warrants follow-up, typically surgical excision. True cysts can occur in Hashimoto's thyroiditis, and cysts arising from adjacent structures (thyroglossal duct cyst, branchial cleft cyst, and parathyroid cyst) can be mistaken clinically for thyroid nodules [10].

CELLULAR FOLLICULAR LESIONS Thyroid aspirates of even non-neoplastic nodules can be remarkably cellular. When the cell to colloid ratio is high, these nodules are best classified as cellular follicular lesions. This generic category encompasses cellular (adenomatous) goiters, hyperplasias, and follicular neoplasms that usually cannot be reliably distinguished by cytomorphology alone [9,10]. Clinicians should correlate cytology findings with their clinical impression to determine whether close follow-up, suppression therapy, repeat aspiration, or surgery is indicated. Hyperplasia, both diffuse (Graves' disease) and solitary (toxic nodule), is difficult to distinguish from a follicular neoplasm using the Papanicolaou stain alone. The Romanowsky (Diff-Quik®) stains accentuate cytoplasmic changes that have been variously named flame cells, fire flares, and colloid suds [9,10,23]. Although not pathognomonic, when prominent, these features are usually indicative of hyperplastic change, and clinical correlation with radioologic study is warranted.

High cellularity aspirates of follicular cells, arranged in large irregular groups, trabeculae or microfollicles, with minimal or absent colloid are very worrisome in follicular neoplasms. The same holds true when pure populations of Hürthle cells are encountered. Currently, follicular neoplasms of the thyroid cannot be definitively separated into follicular adenomas and follicular carcinomas using FNAB. The distinction between cellular follicular lesions and follicular neoplasms is often difficult. Most of these lesions will require histologic evaluation. Typically, FNAB of follicular neoplasms shows a monomorphic population of follicular cells arranged in microfollicles (Fig. 4A). The neoplastic cells have increased nuclear-to-cytoplasmic ratios and distinct nucleoli [9,10,12,24] (Fig. 4B). Similarly, Hürthle cell neoplasms cannot be further differentiated [10, 12,25]. Hürthle cells are very characteristic large polygonal cells with well-defined cell borders, abundant granular cytoplasm, round nuclei, and large, prominent nucleoli (Fig. 5A,B). Hürthle cell lesions are particularly problematic for two reasons. First, they can show significant cytologic atypia yet behave as benign lesions; the converse is also true—relatively bland Hürthle cell nodules may be diagnosed as carcinoma when excised. Hürthle cell carcinomas often show increased nuclear-to-cytoplasmic ratios that can be used to suggest the diagnosis. Second, non-neoplastic Hürthle cell proliferations in Hashimoto's thyroiditis may produce a discrete nodule that, when aspirated, produces a pure population of Hürthle cells that is misdiagnosed as a neoplasm. For this reason, the majority of Hürthle

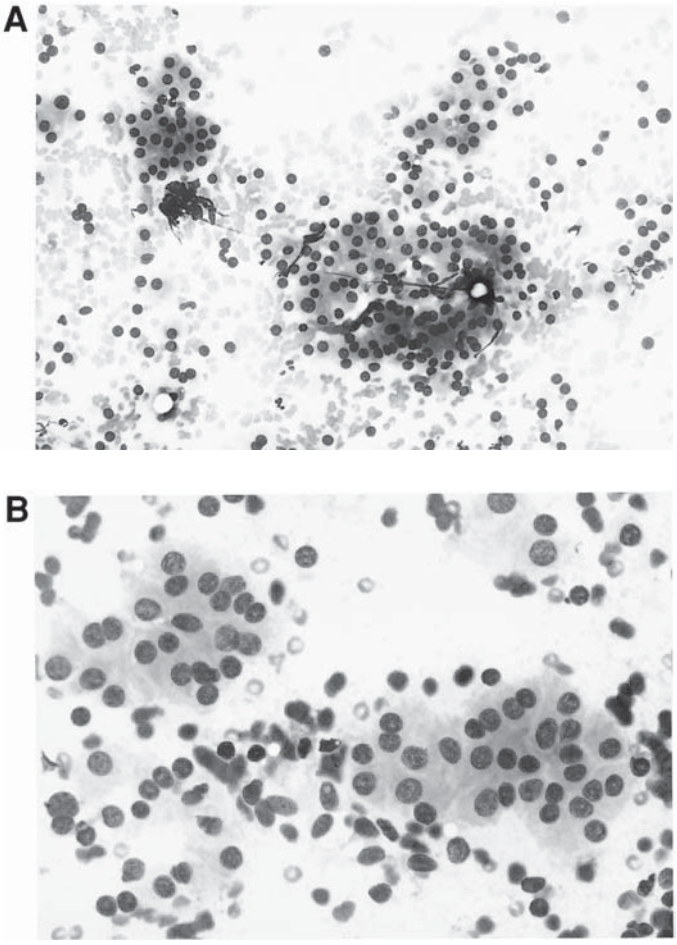


Figure 4 (**A**) Follicular neoplasm. Highly cellular aspirates of follicular neoplasms show individual and groups of disorganized follicular cells. Microfollicles and absent colloid are most indicative of a follicular neoplasm. Diff-Quik® stain, original magnification ×200. (**B**) Follicular neoplasm. These neoplasms frequently display small irregular groups and microfollicles. The cells have increased nuclear-to-cytoplasmic ratios and nucleoli are often visible. Diff-Quik® stain, original magnification ×400.

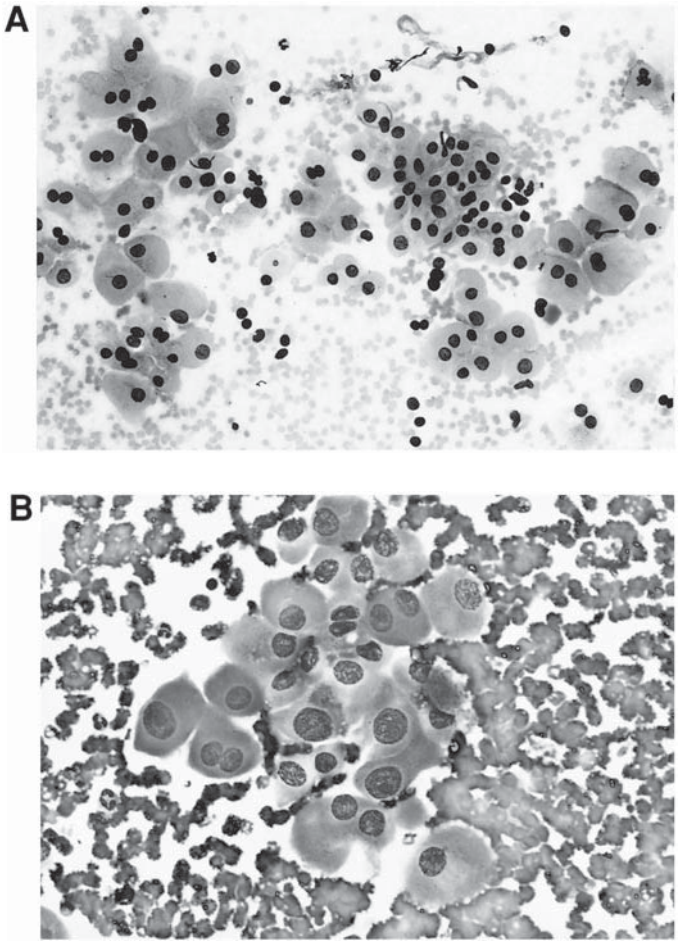


Figure 5 (**A**) Hürthle cell neoplasm. Hürthle cell tumors often show variation in cell as well as nuclear size and binucleation is not uncommon. The biologic behavior of these lesions cannot be predicted by cell atypia. Diff-Quik® stain, original magnification ×200. (**B**) Hürthle cell neoplasm. Cellular aspirates of Hürthle cell tumors contain a pure population of Hürthle cells. These cells are characterized by abundant granular cytoplasm, round eccentric nuclei, and prominent nucleoli. Diff-Quik® stain, original magnification ×400.

cell lesions are surgically excised for a definitive diagnosis. There are several promising ancillary techniques that should soon prove useful in making these distinctions, thereby avoiding unnecessary surgery. Ancillary testing for thyroid aspirates currently includes traditional immunohistochemistry, *in situ* hybridization, cytogenetics, molecular diagnostics, infectious disease testing, and flow cytometry. All of these techniques can be performed on FNAB specimens when the material has been triaged and handled appropriately. In fact, FNAB, particularly when performed by a pathologist–aspirator, provides an optimal specimen for these studies; fresh tissue is obtained, evaluated, and processed immediately in the manner that is most appropriate. A few of the more promising and exciting molecular and protein markers for use in ancillary testing of thyroid gland lesions are discussed below.

THYROIDITIS Inflammatory diseases usually result in diffuse involvement of the thyroid gland (Table 2). As FNAB is the procedure of choice for masses or nodules, sampling becomes an issue in the cytologic diagnosis of thyroiditis. Acute thyroiditis is rarely encountered on aspiration because the clinical presentation is usually diagnostic. The gland is very tender and swollen and, as such, aspiration is extremely painful and the patient will typically allow only one attempt! Follicular cells are scant and often degenerated; the predominant cell type will be neutrophils with bacteria often seen, especially on Diff-Quik® stained preparations [9,10,26,27] (Fig. 6). Subacute or granulomatous (DeQuervain's) thyroiditis is usually self-limited, resolving over several weeks and therefore is not commonly aspirated. As its name implies, the consistent finding is one of degenerated follicular cells with an inflammatory component of histiocytes, multinucleated giant cells, and granulomas [9,10,27–29,30,33,34] (Figs. 7A,B). Colloid is usually scant, while fibrosis and cellular debris may predominate. The inflammatory condition most often typically aspirated is chronic lymphocytic (Hashimoto's) thyroiditis. Follicular cells may be damaged or degenerated, but will still be present in moderate

Table 2
Cytologic Criteria of Thyroiditis

Criteria	*Acute*	*Subacute (DeQuervain's) (granulomatous)*	*Chronic lymphocytic (Hashimoto's)*
Cellularity	Low	Moderate	Moderate to high
Colloid	Absent	Scant to moderate	Scant
Follicular cells	Rare, often degenerated	Few to moderate, degenerated	Moderate, some degeneration
Other cell types	Abundant neutrophils (early stage) Histiocytes, fibroblasts (late stage)	Multinucleated giant cells Epithelioid histocytes Granulomas Mixed inflammatory cells	Hürthle cells Polymorphous population of lymphocytes, germinal centers, plasma cells
Background elements	Bacteria Fibrin	Debris	Lymphoid tangles
Ancillary studies	Microbiology for culture and sensitivity	HLA-B35 HLA-B67	Flow cytometry to exclude lymphoma Serum autoantibodies

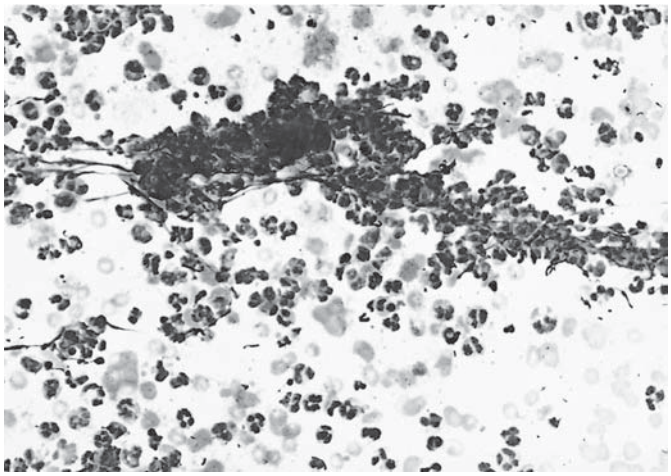


Figure 6 Acute thyroiditis. Numerous neutrophils, fibrin, and cell debris are the predominant features in this diffuse inflammatory process. Papanicolaou stain, original magnification ×200.

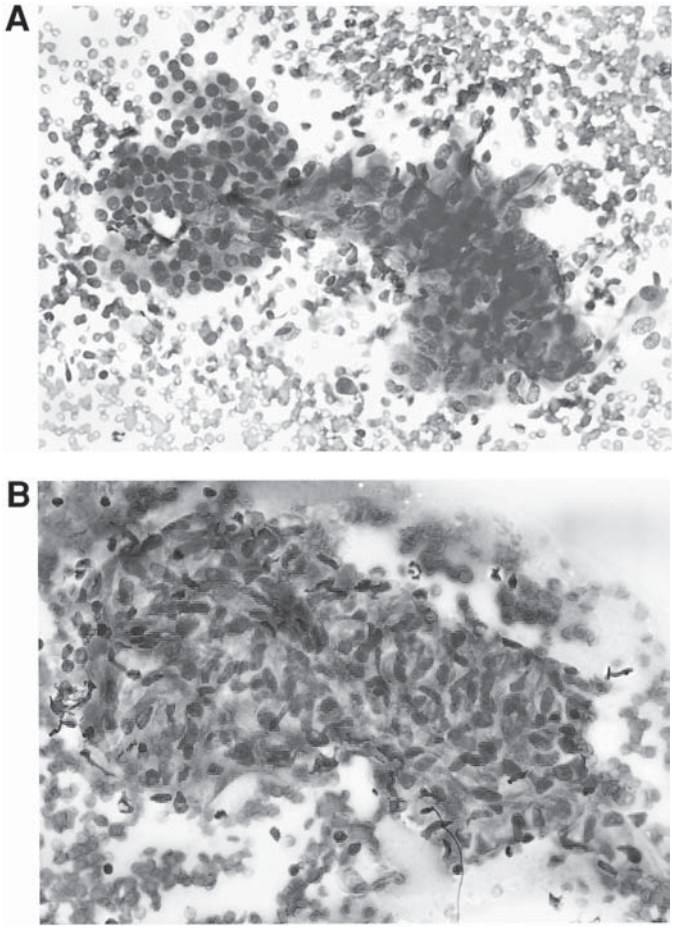


Figure 7 (**A**) Subacute thyroiditis. Aspirate smears from this thyroiditis show an intact follicular cell cluster adjacent to a granuloma. Inflammatory cells and stripped follicular cell nuclei may be seen in the bloody background. Diff-Quik®, original magnification ×200. (**B**) Subacute thyroiditis. High magnification of a single, large non-necrotizing granuloma. Epithelioid histiocyte nuclei are characteristically elongate, giving rise to the term "footprint" nuclei. Diff-Quik®, original magnification ×400.

numbers in aspiration smears (Fig. 8A). A heterogeneous population of lymphocytes, plasma cells, and germinal centers is the predominant inflammatory component. Lymphoid tangles, disrupted lymphocyte DNA, are often encountered (Fig. 8B) Colloid is scant or absent. Hürthle cells are often present as cells scattered singly or in small groups. As discussed above, a diagnostic problem may occur if a palpable Hürthle cell nodule develops, as selective aspiration of this non-neoplastic proliferation may result in a diagnosis of Hürthle cell tumor. Because carcinomas, especially papillary carcinoma, and lymphoma may arise in this condition, sampling by FNAB is critical. Although the cytomorphology alone is usually sufficient to diagnose chronic lymphocytic thyroiditis additional laboratory studies, that is, serum autoantibodies, can help confirm the clinical and cytologic impression.

MALIGNANT NEOPLASMS FNAB is used as a diagnostic procedure for malignant neoplasms of the thyroid. The cytologic criteria are well known and distinctive for the major malignancies (Table 3). Papillary carcinoma is the most frequent malignant diagnosis rendered on FNAB of the thyroid. Specific cytologic criteria exist allowing for very accurate diagnosis of this cancer [9,10,31,32]. The most important of these criteria are the characteristic nuclear grooves and intranuclear cytoplasmic inclusions (Figs. 9A,B). Squamoid cytoplasm and powdery chromatin are also features of the neoplastic cells (Fig. 10). In

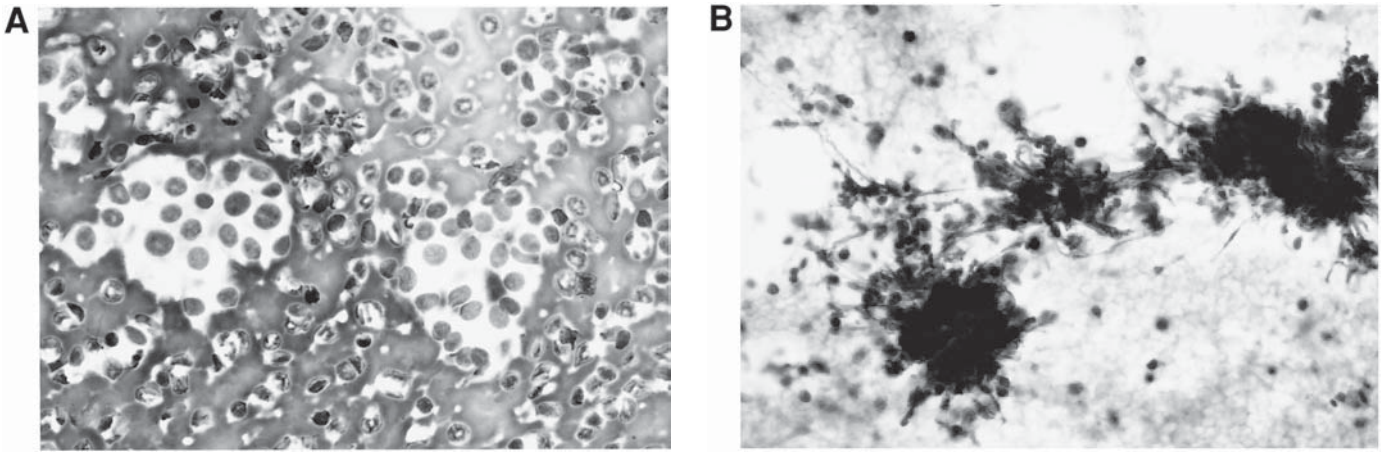


Figure 8 (**A**) Hashimoto's thyroiditis. Moderate to high cellularity smears that contain small clusters of follicular cells, Hürthle cells, and a polymorphous population of lymphocytes. Diff-Quik® stain, original magnification ×400. (**B**) Hashimoto's thyroiditis. In addition to a heterogeneous population of lymphocytes and germinal centers, lymphoid tangles are often seen in chronic lymphocytic thyroiditis. Diff-Quik®, original magnification ×200.

Table 3
Cytologic Criteria of Thyroid Carcinomas

Cytologic criteria	*Papillary carcinoma*	*Medullary carcinoma*	*Anaplastic*
Cellularity	High	High	High
Colloid	Ropy "bubble gum"	Absent	Absent
Pattern	Papillae with fibrovascular cores Monolayer sheets	Discohesive Occ. papillary	Pleomorphic, obviously malignant cells
Cytomorphology	Monomorphic follicular cells Squamoid cytoplasm Nuclear grooves and inclusions Powdery chromatin	Variable morphology: plasmacytoid, Hürthle-like, spindle-shaped carcinoid-like Neuroendocrine chromatin Neurosecretory granules	Extreme cytologic atypia High N/C ratio Prominent nucleoli Atypical mitoses
Background Elements	Multinucleated giant cells Psammoma bodies Occ. cystic degenaration	Amyloid	Neutrophils Debris Necrosis
Ancillary Studies	Thyroglobulin, TTF1 Galectin -3 *RET/PTC* gene rearrangement	Congo red-amyloid Calcitonin Genetic testing: MEN 2A, 2B Overexpression of *RET* protooncogene	Thyroglobulin-negative

addition, a low-magnification survey of the aspirate smear will reveal abundant cells arranged in flat sheets or as papillary fronds (Fig. 11). Stripped fibrovascular cores with adjacent follicular cells may also be present. Background elements that are also suggestive of papillary carcinoma include dense ropy colloid, multinucleated giant cells, and psammoma bodies.

Medullary carcinoma is one of the more challenging diagnoses to make by aspiration cytology as aspirates may reveal variable tumor cell cytomorphology: plasmacytoid, classic neuroendocrine (carcinoidlike), Hürthe cell-like, and spindle-cell [9,10,33–35] (Fig. 12A,B). Intranuclear inclusions may be present but the tumor cells generally lack the monotony of Hürthle cell tumors and papillary carcinomas. Amyloid, when present, may resemble the dense, ropy colloid characteristic of papillary carcinoma. Fortunately, medullary carcinomas derive from C cells and as such may have cytoplasmic neurosecretory granules visible as small perinuclear red granules on Diff-Quik® stains (Fig. 12C). In addition, calcitonin can be used to confirm this diagnosis.

Aspiration smears of anaplastic carcinoma reveal obvious pleomorphic malignant cells usually with an accompanying neutrophilic infiltrate, atypical mitoses, and necrosis [9,10,36–38] (Fig. 13). The differential diagnosis is usually clinical because these carcinomas present in elderly patients as rapidly enlarging masses that often comprise breathing. This presentation is also typical of high-grade malignant lymphomas of the thyroid (Fig. 14). The distinction between the two entities is not a problem cytologically. The benefit of FNAB in these cases is the rapid identification of malignancy such that patients may be immediately triaged to radiation oncology.

Metastases can present as thyroid masses from distant primaries or as direct extensions of tumors from nearby structures, particularly squamous cell carcinoma, and less commonly, adenoid cystic carcinoma of the trachea. Renal cell carcinoma is

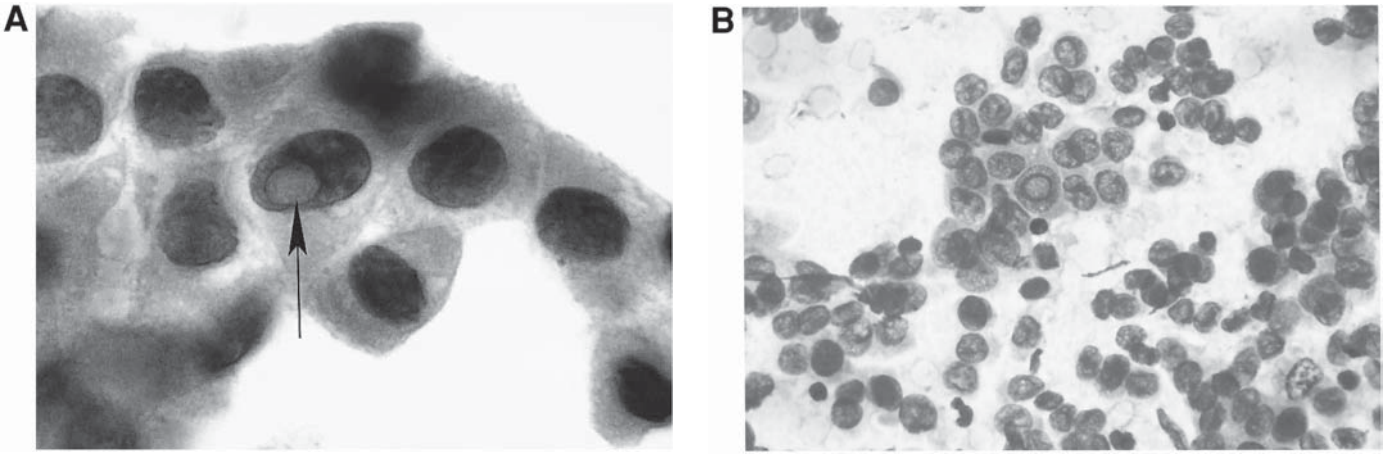


Figure 9 (**A**) Papillary carcinoma. Neoplastic follicular cells have well defined cell borders, squamoid cytoplasm, and often large intranuclear cytoplasmic inclusions. Papanicolaou stain, original magnification ×1000. (**B**) Papillary carcinoma. Scattered neoplastic follicular cells with prominent nuclear grooves and a solitary intranuclear cytoplasmic inclusion. Papanicolaou stain, original magnification ×400.

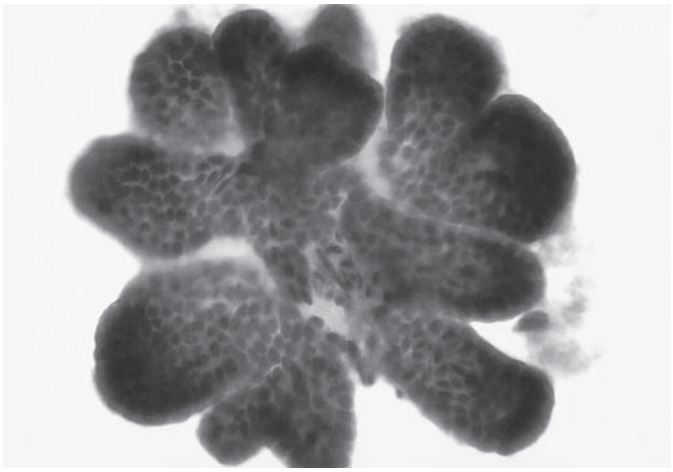


Figure 10 Papillary carcinoma. Papillae, fibrovascular cores covered by neoplastic follicular cells, may be aspirated intact and appear as three-dimensional structures on Papanicolaou stained smears. Papanicolaou stain, original magnification ×200.

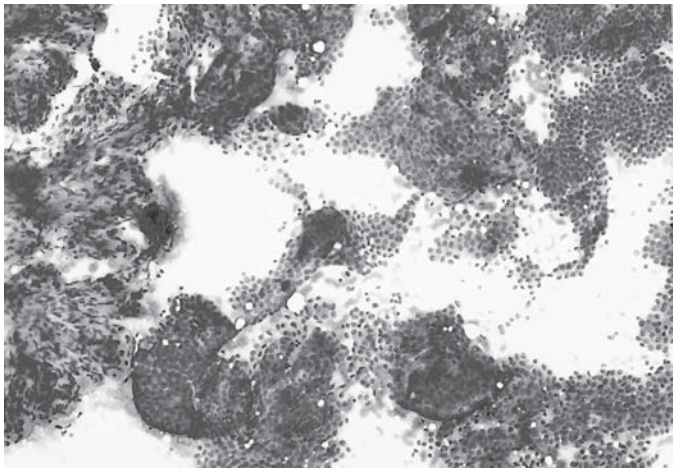
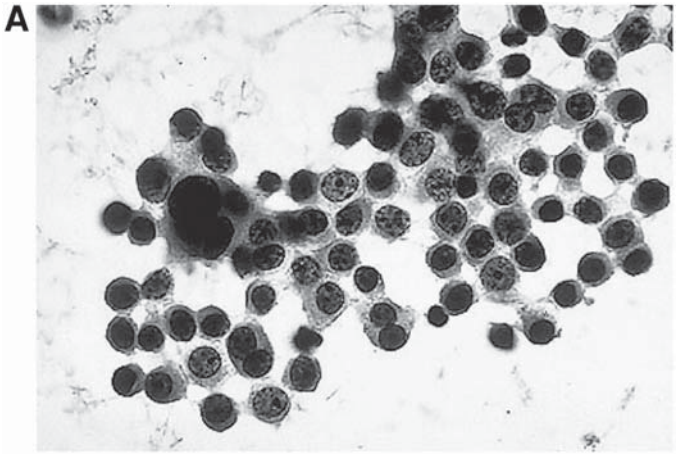


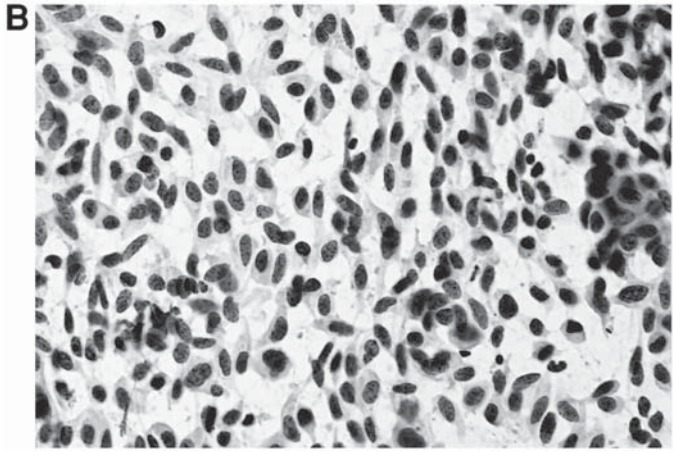
Figure 11 Papillary carcinoma. Highly cellular aspirates with naked fibrovascular cores and detached, often furled sheets of follicular cells are low-magnification features of papillary carcinoma. Papanicolaou-stain, original magnification ×100.

particularly difficult to distinguish from histiocytes. Cytologic clues include the delicate, often frayed cytoplasm and microvesicular smear background of renal cell carcinomas (Fig. 15). Clinical history and review of prior pathology play a key role in the workup of any aspiration and are especially important when dealing with the possibility of metastases.

Occasionally, FNAB will create changes in the thyroid that can complicate a histologic diagnosis following excision [39–44]. These changes are most frequently known as "worrisome histologic alterations following fine needle aspiration of the thyroid" or WHAFFT. The incidence of these characteristic changes varies, but is usually reported to be <40%. Changes can include hemorrhage, granulation tissue, granuloma formation, cyst formation, papillary degeneration, infarction, necrosis, capsular distortion (pseudoinvasion), random nuclear atypia, mitoses, nuclear clearing, fibrosis, metaplasia (oncocytic, spindle cell, and squamous), and calcification. Of all of these alterations, the most significant are infarction and capsular distortion, as they can make the diagnosis of malignancy and/or true invasion more challenging. Fortunately, these changes are uncommon; however, it is helpful to recognize the overall pattern of WHAFFT, and to require the presence of true vascular invasion when making a diagnosis of follicular carcinoma or Hürthle cell carcinoma. Awareness of previous FNAB results is an essential component of the histologic diagnosis of a lesion with extensive infarction. Of thyroid lesions, Hürthle cell lesions are most likely to become infarcted, either spontaneously or as the result of fine needle aspiration.

***RET* Protooncogene** Germline mutations of the *RET* protooncogene are responsible for medullary carcinoma associated with multiple endocrine neoplasm (MEN) syndromes, and *RET/PTC* gene rearrangements account for a significant number of papillary thyroid carcinomas [45,46]. *RET* alterations can be identified by multiple modalities. RET protein (or lack of protein





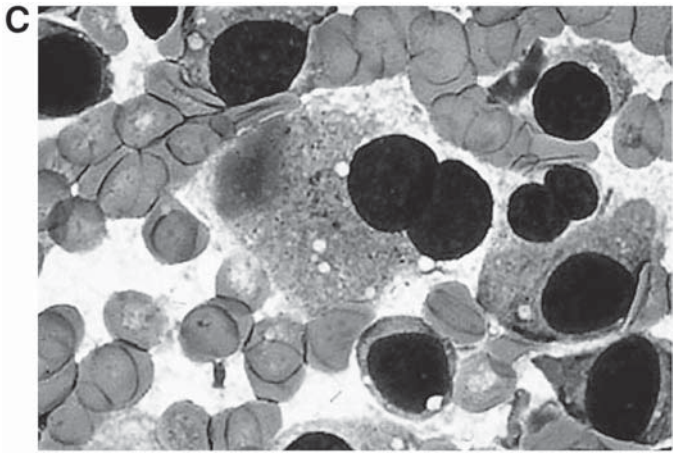


Figure 12 (**A**) Medullary carcinoma. Aspirates of this malignancy are highly cellular, typically with a discohesive population of cells with variable pleomorphism. Bi- and multinucleation may be seen. Diff-Quik® stain, original magnification ×200. (**B**) Medullary carcinoma. The spindle cell variant of medullary carcinoma presents as highly cellular aspirates with discohesive spindle cells that have scant cytoplasm and elongate oval hyperchromatic nuclei. Diff-Quik® stain, original magnification ×200. (**C**) Figure 12 Medullary carcinoma. Neurosecretory granules are seen adjacent to the nucleus. These appear as red granules on Diff-Quik® stain. Diff-Quik® stain, original magnification ×400.

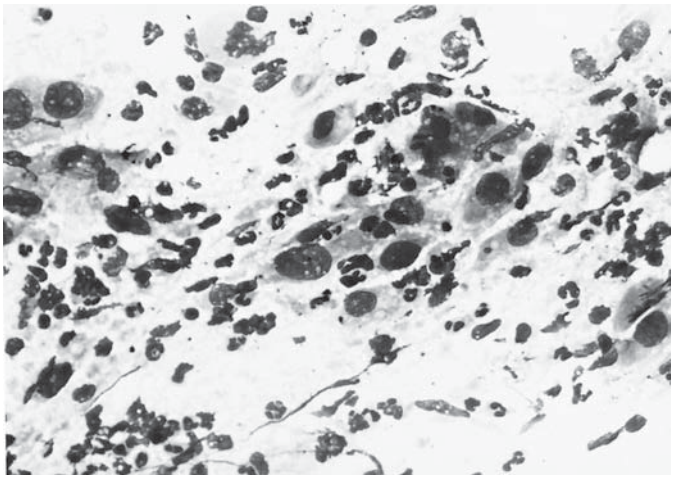


Figure 13 Anaplastic carcinoma. Cellular aspirate with numerous pleomorphic malignant cells with high nuclear-to-cytoplasmic ratios. Necrosis and neutrophils are frequent background elements. Emperipolesis is often seen. Diff-Quik® stain, original magnification ×200.

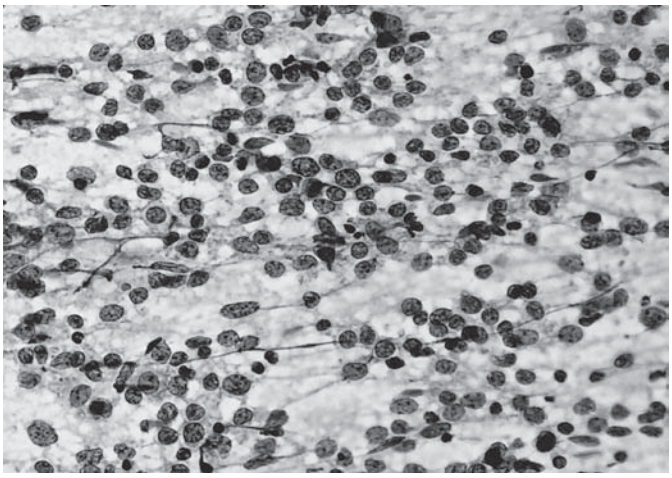


Figure 14 Non-Hodgkin's lymphoma. Highly cellular aspirate smears with monomorphic population of neoplastic lymphocytes. The diagnosis of lymphoma of the thyroid gland uses the same cytologic criteria as those for lymph node. DNA artifact, strings of nuclear material flowing in the direction of the smear, is common owing to the fragility of the tumor cells. Papanicolaou stain, original magnification ×200.

expression) can be detected by immunocytochemistry. Specific inherited *RET* mutations can be identified with polymerase chain reaction (PCR) and sequencing reactions. *RET/PTC* can be detected by reverse transcriptase-PCR (RT-PCR) or by interphase fluorescence *in situ* hybridization (FISH) [45–47]. Efficient detection of the gene rearrangement in FNAB material by RT-PCR analysis has been documented and was shown to improve the ability to make a definitive diagnosis of malignancy on a limited specimen [48]. *RET/PTC* rearrangements are thought to be specific for papillary carcinoma, although mutations have been reported to occur in Hashimoto's thyroiditis and follicular adenomas [45,49]. The biologic meaning of this finding is not clear at this time. Some Hürthle cell tumors show *RET/PTC* mutations. These tumors may represent a new subclassification

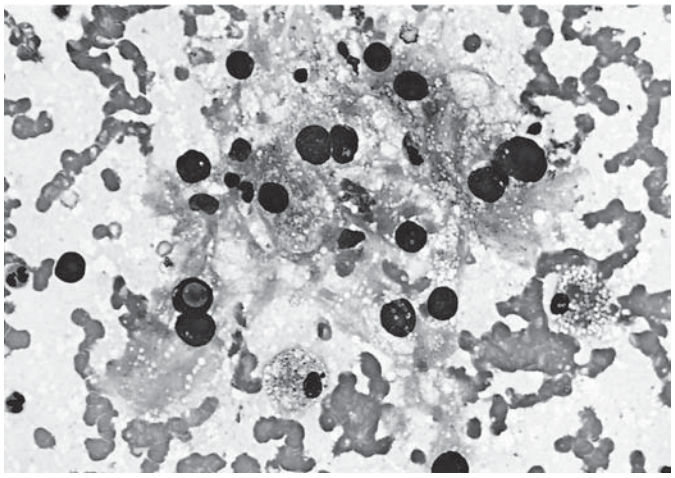


Figure 15 Metastatic renal cell carcinoma. Often cellular, these metastases show relatively bland cells with abundant vacuolated cytoplasm and round regular nuclei. Occasionally intranuclear inclusions can be seen. The cytoplasm is often detached from these fragile cells when smears are performed, lending a microvesicular appearance to the smear background. Diff-Quik® stain, original magnification ×200.

category of Hürthle cell tumors known as Hürthle cell papillary thyroid carcinoma [50].

***ras* Oncogene** Mutations of the *ras* oncogene have been found in most thyroid tumor types, including follicular adenomas and carcinomas [51,52]. These mutations are implicated in the early stages of carcinoma development, and are thought to be important events in the progression to poorly differentiated thyroid carcinoma [51,53]. At least one study has documented the utility of FNAB with PCR and gene sequencing of the *ras* oncogene to diagnose follicular carcinoma [54].

E-Cadherin E-cadherin, a cell surface adhesion molecule, is one of the most promising candidates for distinguishing between adenoma and carcinoma in follicular and Hürthle cell lesions. E-cadherin protein expression, as measured by immunocytochemistry, is reduced in some follicular carcinomas and Hürthle cell carcinomas [55–60]. The pattern also changes from membranous to cytoplasmic. These changes have been found to correlate with prognosis. The assays can be performed on FNAB material with acceptable results [60].

Galectin-3 Galectin-3 is another marker that could aid in the differentiation between benign and malignant thyroid lesions. Present almost exclusively in malignant lesions, galectin-3 protein expression in FNAB material has been promoted as a tool to increase the accuracy of preoperative evaluation of thyroid lesions [61–64]. Some have even suggested that sparsely cellular aspirates of cystic lesions that are positive for galectin-3 should be considered diagnostic of cystic papillary carcinoma and should be surgically excised [65]. In addition, the assay has been shown to be useful in the differential diagnosis of FNAB of Hürthle cell lesions [66], and in the diagnosis of medullary carcinoma (vs C-cell hyperplasia, in patients with familial medullary carcinoma or MEN) [67].

Ki-67 and Cyclin D1 Ki-67 and cyclin D1 are two more molecular markers that, particularly when they are evaluated together, can aid in the differentiation between adenoma and carcinoma in Hürthle cell neoplasms. These two markers, assayed primarily through immunocytochemical techniques, have been shown to be predictive of carcinoma vs benign tissue, and indicative of tumor invasiveness and aggressiveness, and have been reflective of overall patient survival [68–70].

Loss of Heterozygosity Microsatellites are one of the most useful molecular markers currently employed to evaluate genetic changes in tumors. They are small regions of DNA containing a variable number—two to five— of nucleotide repeats, typically found in close proximity to a known gene of interest. The length of the microsatellite region is specific to each inherited allele. Therefore, a significant number of patients will have two differently sized microsatellite regions at any given location in their normal tissue. When that tissue becomes neoplastic, one allele is frequently lost, converting the normal heterozygous pattern to an abnormal homozygous pattern. Of course, when the pattern is homozygous to begin with, the test is noninformative. From microdissected FNAB material, microsatellite regions can be easily amplified by PCR and accurately sized using electrophoresis.

Numerous microsatellite regions have been evaluated for loss of heterozygosity (LOH) in an attempt to define neoplasia and malignancy and also to determine prognosis. Several are relevant to thyroid tumors. LOH has been noted in anaplastic thyroid carcinoma within chromosomes 1q, 7q, 9p, 11p, 16p, 17p, 18q, 19p, and 22q. LOH at some of these sites can be seen in more differentiated thyroid tumors and may indicate more aggressive tumor behavior [12,71–73]. LOH at the *p16* gene locus on chromosome 9p has been associated with papillary thyroid carcinoma [12]. LOH on chromosome 10q, at the *PTEN* gene locus, and at 11q, has been identified in follicular adenomas and carcinomas of the thyroid, while LOH within chromosomes 2, 3p, 7q, 9p (the *p16* gene locus), and 22q has been associated with follicular carcinomas [12,74]. LOH at the *p53* gene locus on 17p has been associated with metastatic dissemination of follicular carcinoma [12]. Hürthle cell adenomas have been associated with LOH on chromosomes 3q, 10q, and 18q, while LOH on chromosomes 1q and 2p is more common in Hürthle cell carcinomas [12].

Telomerase Multiple studies have examined telomerase activity and human telomerase reverse transcriptase (*hTERT*) gene expression as markers of malignancy in FNAB of the thyroid with encouraging results [75–78]. One study also showed prognostic value associated with telomerase immunostaining [79]. However, a recent study examining the effectiveness of telomerase evaluation of FNAB specimens found that the assay did not provide additional information and that the assay could be positive in some inflammatory lesions [80]. Nonetheless, this marker does appear to be useful and could be used to determine malignancy in FNAB specimens, possibly in combination with other markers.

p53 Mutations of the *p53* tumor suppressor gene have been consistently documented in anaplastic carcinoma of the thyroid and linked to dedifferentiation of papillary, follicular, and insular thyroid carcinomas [81–83]. These mutations can be assessed by direct sequencing reactions, or by measuring the characteristic protein overexpression in the tissue by immunohistochemistry. Mutations of *p53* in anaplastic carcinoma of the

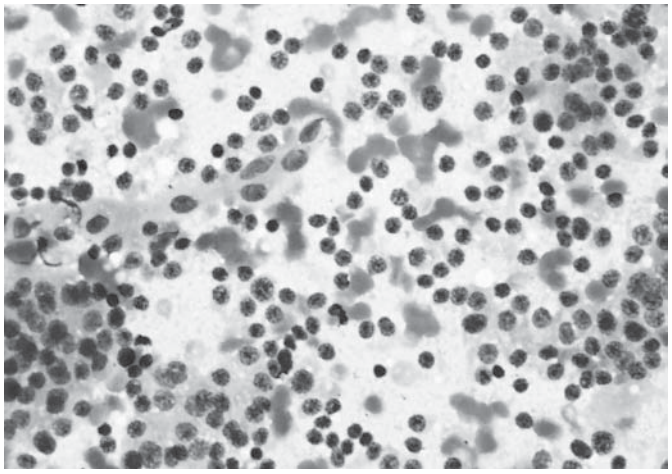


Figure 16 Parathyroid gland. Aspirates from parathyroid lesions are highly cellular with a dispersed population of small delicate cells with round nuclei and indistinct nucleoli. Stripped nuclei are abundant. Diff-Quik® stain, original magnification ×200.

thyroid are promising targets for gene therapy, particularly in combination with traditional chemotherapeutic agents [84–86].

PARATHYROID

The primary indication for FNAB of the parathyroid gland is the presence of a palpable neck mass [10,87]. Usually a parathyroid lesion is but one diagnosis in a clinical differential that includes thyroid and possibly lymph node lesions. FNAB is very useful in distinguishing thyroid from parathyroid. In addition, ultrasound-guided FNAB of suspected parathyroid tissue in patients with known hyperparathyroidism has been shown to be an effective technique for localizing the abnormal parathyroid tissue preoperatively, particularly if the patient has had prior neck surgery [88–90]. Parathyroid tissue is also occasionally aspirated when it is encompassed by thyroid tissue and presents clinically as a thyroid mass. As in thyroid aspirations, relative contraindications to FNAB of the parathyroid glands include a recent history of anticoagulant therapy or the presence of a bleeding diathesis, due to the risk of hematoma formation. When parathyroid cysts are aspirated, water-clear fluid is obtained [10,91]. These cysts usually have very few, if any, cells to evaluate. However, the fluid can be sent for chemical analysis; the extremely high level of parathyroid hormone (PTH) will confirm the diagnosis.

It is very important to be able to recognize the normal morphology of the parathyroid, particularly to distinguish it from thyroid. This is the first, and frequently critical step, in narrowing the differential diagnosis. Immunochemistry for PTH can also be useful in this scenario [90]. Aspirates of parathyroid are moderately cellular with small cuboidal cells with scant delicate cytoplasm (Fig. 16). The chief and clear cells that predominate in most aspirate smears are cytomorphologically similar with pale blue or clear cytoplasm. Their nuclei are smaller than those of thyroid follicular cells, and they tend to form loose aggregates and rosettelike structures rather than distinct follicles. Colloid is not present. The cytoplasm of clear cells is fragile and easily disrupted during smear preparation; as a result numerous stripped nuclei may be scattered throughout the smear. As with follicular lesions of the thyroid, parathyroid hyperplasia, adenoma, and carcinoma cannot reliably be distinguished by FNAB [10,87,92]. However, offering this differential within the particular clinical context is usually sufficient to plan definitive surgical therapy [93,94]. Parathyroid carcinoma should be suspected when aspirates show pronounced cellularity, increased numbers of single cells with marked cellular anaplasia, and necrosis [95,96]. However, the definitive diagnosis of carcinoma rests on irrefutable histologic evidence of local invasion or metastasis.

Molecular diagnostic techniques may soon prove useful in refining these distinctions. LOH has already been noted in parathyroid adenomas on chromosomes 1p, 11q, and 13q [12,97–99], and in parathyroid hyperplasia on chromosomes 2, 7p, and 18q [100].

ADRENAL

The primary indication for FNAB of an adrenal gland is the presence of a radiologically detected mass that is clinically suspicious for malignancy. Frequently, adrenal lesions are noted incidentally on an abdominal CT scan performed for an unrelated reason. Suspicious lesions are usually solid and >2 cm. Relative contraindications include a recent history of anticoagulant therapy or the presence of a bleeding diathesis, due to the risk of hematoma formation. One absolute contraindication is suspicion of pheochromocytoma, due to the risk of catecholamine crisis and/or fatal hemorrhagic complications. Still, unsuspected pheochromocytomas are usually aspirated without incident. The sensitivity and specificity of FNAB of the adrenal gland for the diagnosis of malignancy are both >90% [101–103].

Once an adrenal mass is identified, FNAB is commonly used to determine if the patient has a primary adrenal cortical lesion or a metastasis. Metastatic disease is one of the most frequent diagnoses encountered in aspiration of adrenal glands. Distinguishing primary from metastatic disease is usually straightforward, especially if the primary disease is known and histology or cytology is available for review. At times, however, it can be difficult to differentiate a highly dysplastic adrenal cortical carcinoma from some metastatic lesions based on cytomorphology alone. Another pitfall occurs if the stripped nuclei of normal adrenal gland or cortical lesions are abundant. This pattern can be misinterpreted as small cell carcinoma. In aspirate smears, the recognition of cell molding, individual cell necrosis, and lack of microvesicular background support a diagnosis of metastatic small cell carcinoma.

When the mass is identified as primary adrenal cortical lesion, radiologic and clinical information must be integrated with cytomorphology. Adrenal cortical cells are typically polygonal, clustering in small groups or cords with central round to oval nuclei. Occasional endocrine atypia manifests as enlarged, hyperchromatic nuclei and morula formation [103–105]. The amount of cytoplasm varies within the different zones of the cortex. Cortical cells from the zona glomerulosa and fasiculata tend to have delicate vacuolated, clear cytoplasm with stripped nuclei, while cells from the zona reticularis are more compact with granular cytoplasm, often with lipofuscin pigment (Fig. 17).

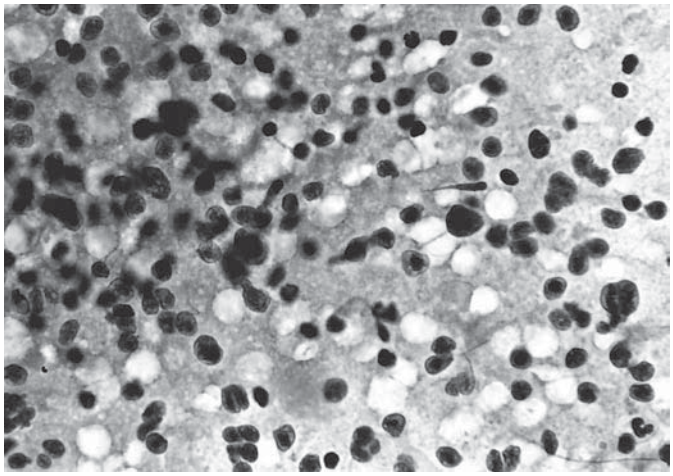


Figure 17 Adrenal cortical lesion. Aspirates from cortical lesions, both hyperplasia and neoplasms, are often indistinct from aspirates of normal adrenal. Aspirates are usually highly cellular with cortical cells dispersed singly or in small groups. The cells have moderate amounts of vacuolated cytoplasm that is easily stripped from their nuclei; the latter appear scattered throughout the background. Diff-Quik® stain, original magnification ×200.

Table 4
Cytologic Criteria of Selected Adrenal Lesions

Cytologic criteria	*Cortical adenoma*	*Cortical adenocarcinoma*	*Pheochromocytoma*
Cellularity	Moderate to high	Moderate to high	High
Pattern	Discohesive Loose groups Stripped nuclei	Predominantly discohesive Loose groups Stripped nuclei	Discohesive Loose groups
Tumor cells	Bland	Bland to moderate atypia Mitoses	Marked polymorphism Distinct nucleoli Occ. mitoses
Background	Microvesicular (lipid)	Microvesicular (lipid) Occ. necrosis	Bloody
Ancillary	Melan-A α-Inhibin	Melan-A α-Inhibin	Chromogranin, synaptophysin Genetic testing for MEN Von Hippel–Lindau syndrome

As with thyroid and parathyroid, the differentiation of adrenal hyperplasia from adenoma and carcinoma is very difficult, if not impossible, by FNAB. Adrenal cortical hyperplasia typically presents as a cellular specimen with unremarkable adrenal cortical cells dispersed singly or in groups with scattered stripped nuclei. The dispersed cytoplasm lends a microvesicular appearance to the smear background as a result of the presence of lipids. Mitotic figures and necrotic debris are absent. Well circumscribed or encapsulated nodules that are aspirated and composed of adrenal cortical cells typically represent a neoplasm. Adenomas are cytologically identical in appearance to aspirates from normal and hyperplastic adrenal glands (Table 4). Although one can use radiologic data as a clue to the biologic behavior, cytologic evidence from carcinoma that is worrisome include the presence of necrosis, diffuse cytologic atypia, and mitoses, especially atypical forms [106]. These lesions typically must be excised surgically for a more comprehensive assessment.

Primary lesions of the adrenal medulla, pheochromocytomas, are rarely aspirated, often because the clinical presentation in conjunction with laboratory studies (increased vanillylmandelic acid and catecholamines in urine and/or plasma) and radiologic studies abrogate the need to risk a hypertensive crisis to obtain a confirmatory aspiration/biopsy. However, when aspirated, smears are usually hypercellular with the neoplastic cells arranged singly or in loose clusters (Fig. 18A). The cells demonstrate considerable polymorphism and anisonucleosis. Cytoplasm is often frayed and stripped nuclei may be present. Nucleoli are prominent, but mitoses infrequent (Fig. 18B). Nuclear inclusions may be seen occasionally [107]. Some cases of pheochromocytoma may show extensive pleomorphism, while others may have a more obvious neuroendocrine appearance.

The third most common malignancy of childhood, neuroblastoma, arises from adrenal and other sites containing sympathetic neural tissue. Aspirates of neuroblastomas are hypercellular with small hyperchromatic cells scattered singly, in molded clusters, or in loose Homer–Wright rosettes [108]. The cells have a high nuclear-to-cytoplasmic ratio with dispersed, granular "neuroendocrine" chromatin and scant fragile cytoplasm (Fig. 19A). Intact rosettes will often show a fine fibrillar material centrally, neuropil that stains a vivid pink or magenta on smears prepared with Diff-Quik®. Necrosis, mitoses, and rarely calcification are other features that may be identified. The differential diagnosis for aspirates with this cytomorphology can

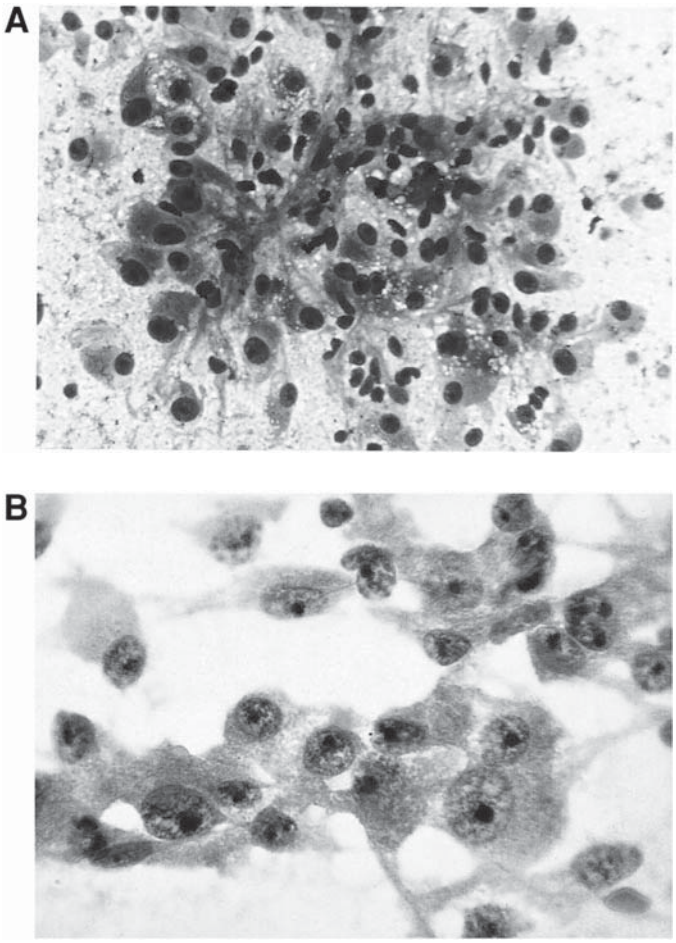


Figure 18 (**A**) Pheochromocytoma. Aspirates are usually cellular with the neoplastic cells arranged in loose clusters with moderate amounts of delicate cytoplasm. Diff-Quik® stain, original magnification ×200. (**B**) Pheochromocytoma. Moderate to large neoplastic cells with abundant cytoplasm, large nuclei with granular chromatin and large nucleoli. Papanicolaou stain, original magnification ×400.

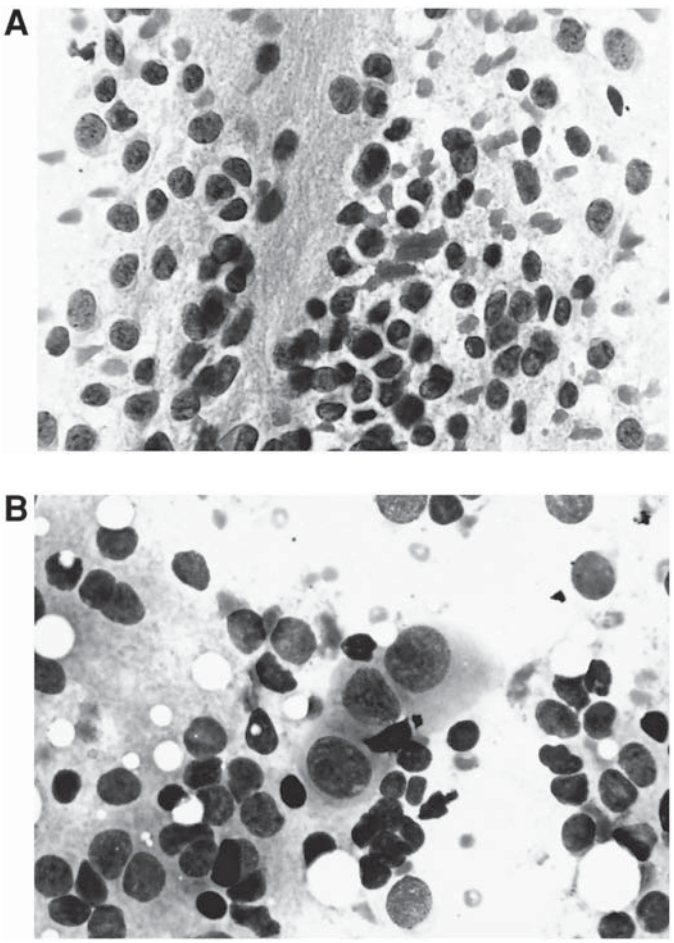


Figure 19 (**A**) Neuroblastoma. Aspirates of this small round blue cell tumor are highly cellular, composed of small round cells with high nuclear-to-cytoplasmic ratios and hyperchromatic nuclei. A delicate fibrillar material, neuropil, may be seen. Diff-Quik® stain, original magnification ×200. (**B**) Ganglioneuroblastoma. The spectrum of neuroblastoma and more differentiated forms rests on the identification of ganglion cells. These are large cells with moderate amounts of cytoplasm, granular chromatin, and distinct nulceoli. Papanicolaou stain, original magnification ×400.

include other pediatric small round blue cell tumors such as Wilms' tumor, primitive neuroectodermal tumor, Ewing's sarcoma, rhabdomyosarcoma, as well as malignant lymphomas. Immunochemistry (neuron-specific enolase [NSE], S100, neurofilaments, leukocyte common antigen [LCA], actin, desmin) as well as DNA ploidy are helpful. Interphase FISH for 1p deletion and N-*myc* amplification for prognosis can also be assessed if enough sample is obtained. The appearance of ganglion cells within aspirates shifts the differential diagnosis toward ganglioneuroblastoma and ganglioneuroma [109]. Ganglion cells are characteristically large cells with moderate amounts of cytoplasm; usually coarse, granular chromatin with distinct nucleoli; and occasional multinucleation (Fig. 19B). Ganglioneuroblastomas show ganglion cells dispersed singly or in small foci throughout the neuroblastomatous component, whereas aspirates of ganglioneuromas have a mixed population of mature ganglion cells, spindle cells and matrix of Schwannian origin, and collagen. As the diagnosis of ganglioneuroma relies on the absence of neuroblasts, and requires adequate sampling, the diagnosis of ganglioneuroma is usually suggested but not definitive on aspirate smears.

MELAN A AND α-INHIBIN Ancillary testing is somewhat limited at this time for adrenal aspirates. The most useful markers in identifying a tumor of adrenal origin (or excluding a metastatic tumor) are melan A103 and α-inhibin [110,111]. Although these markers are not entirely specific for adrenal tissue, they are usually quite helpful, particularly when used in tandem.

LOH FOR ADRENAL CORTICAL LESIONS It is usually impossible to reliably separate adrenal cortical hyperplasia, adenoma, and carcinoma solely based on morphology, sometimes even in the excised surgical specimen. Unfortunately, a dependably characteristic protein marker is not yet available to make this distinction, but LOH has proven to be contributory in many cases.

LOH associated with adrenal cortical carcinoma has been identified on chromosomes 1p, 2p, 3p, and 17p, and LOH iden-

tified on chromosomes 9p (*p16*) and 11q is more common in carcinoma than in adenoma [12,112]. Of note, LOH studies performed on FNAB material obtained from primarily metastatic lesions produced reliable results, which were shown to correlate precisely with the surgical material from the primary tumor [112].

LOH IN PHEOCHROMOCYTOMA Similarly, in pheochromocytoma, it is often impossible to determine malignancy based on morphology alone, sometimes even in the excised surgical specimen. Again, a dependably characteristic protein marker is not yet available to make this distinction. LOH has been noted in pheochromocytomas on chromosomes 1p and 22q [12], but to our knowledge there have been no markers that can differentiate benign from malignant pheochromocytomas as yet.

MARKERS OF PROGNOSIS IN NEUROBLASTOMATOUS TUMORS In neuroblatomatous tumors, ancillary testing for DNA ploidy analysis and for N-*myc* amplification and 1p deletion is quite helpful in determining prognosis and predicting response to therapy. Multiple methodologies are available for performing this testing, most recently including interphase FISH [113].

SUMMARY

FNAB is a simple, rapid, accurate, economical, and minimally invasive technique for the diagnosis of endocrine lesions. Good technical training and adequate experience in obtaining and preparing samples are critical to the overall effectiveness of the procedure. With adequate knowledge of the morphology, supplemented by knowledge access to appropriate ancillary techniques, many diagnoses can be made without resorting to surgical intervention. As technology continues to evolve rapidly, and more molecular and proteomic markers are identified, ancillary techniques will become widely available and many of the current ambiguities in the diagnosis of the most common endocrine lesions will be clarified. The specific information obtained from the FNAB specimen will continue to save more and more patients from invasive, costly procedures. The combined use of FNAB and molecular diagnostics has a great potential to improve patient care.

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18 Neuroendocrine Differentiation Patterns in Various Organs (Including Lung, Breast, Skin, and Urogenital Tract)

MARCO VOLANTE, MD, ANNA SAPINO, MD, MAURO PAPOTTI, MD, DONATELLA PACCHIONI, MD, AND GIANNI BUSSOLATI, MD, FRCPATH

INTRODUCTION

Neuroendocrine (NE) differentiation implies production, storage, and release of appropriate peptide hormones and biogenic amines, acting on target cells through specific receptors via endocrine, paracrine, or autocrine pathways [1]. NE cells constituting the diffuse NE system are present in various organs, dispersed among exocrine cells. They were first extensively investigated in the gastrointestinal tract and pancreas, initially by silver-staining procedures and ultrastructural analysis, and it was soon realized that they have common characteristics, both structural (such as cytoplasmic neurosecretory granules and clear vesicles) and functional [2]. The latter constitutes a vast array of markers, which can now be traced by immunocytochemical procedures, thus acquiring diagnostic interest.

In the gastroenteropancreatic (GEP) area and in the thyroid, different NE cell types are known to exist, each devoted to the production of specific hormones. Advances in the understanding of NE differentiation pathways in these organs led to the identification of NE cells in other organs such as lung, breast, skin, and urogenital tract.

In parallel with advances in determining the presence and significance of NE cells in various organs, NE tumors were described and classified. Although some specific properties have been described in NE tumors of the GEP area and thyroid, all NE tumors have common features, both structural (presence of neurosecretory granules and clear vesicles) and functional, as expression of NE markers acquires diagnostic significance. It was soon realized that NE tumors do not constitute a single, uniform entity, but there is a spectrum of tumors in which the degree of NE differentiation matches a more indolent (benign) behavior. Tumor entities such as carcinoids, well-differentiated NE tumors, and NE carcinomas could thus be established in different organs.

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Appropriate diagnostic and classification criteria, of high clinical and prognostic value, have been established for NE tumors of the GEP area and thyroid (see specific chapters). Similar criteria have also been applied to tumors arising in other organs, although a well-established classification is still lacking. Moreover, besides occasional findings, we still do not know which specific hormones, if any, are being consistently produced by NE cells and tumors of lung, breast, skin, and urogenital tract.

As a consequence, only "common" NE markers are of diagnostic significance in the definition and identification of NE tumors in these organs (Table 1). Among such markers, of major interest are chromogranins/secretogranins, a family of soluble proteins that represent the predominant constituent by weight of neurosecretory granules. The specific function of these proteins is unknown, but endocrine–paracrine effects of fragments of these proteins have been described [3]. Detection of these proteins by specific antibodies or of their expression machinery (mRNA) by molecular biology procedures is not only of diagnostic importance, but also provide information on cell metabolism and on the storage or release of neurosecretory granules. Chromogranin A (CgA), the most widely and intensely expressed member of the family, is stored in large amounts in the cytoplasm of well-differentiated NE tumors (carcinoids), where it is detected by immunocytochemical (ICC) techniques even without adoption of antigen-retrieval procedures. The use of antigen-retrieval techniques is necessary to detect minimal CgA deposits in NE carcinomas.

This chapter discusses NE differentiation patterns in areas outside the GEP and the thyroid, and specifically in (1) lung, (2) breast, (3) skin, and (4) prostate and urothelium.

LUNG

NE CELLS IN NORMAL LUNG AND IN NON-NEOPLASTIC CONDITIONS NE cells (also called Kulchitsky or K cells) of the lung are part of the diffuse NE system, are of

Table 1
Neuroendocrine Cell Markers

Fluorogenic amine content
Amine precursor (5-hydroxytryptophan and DOPA) uptake
Aromatic amino acid decarboxylase
Nonspecific esterase or cholinesterase
α-Glycerophosphate deydrogenase
Peptide hormone synthesis
Voltage-dependent Ca^{2+} or Na^{2+} channels
Electrical excitability
Neuron-specific enolase
Chromogranins and secretogranins
Neuroendocrine secretory protein (NESP55)
Chromomembrin B
Synaptophysin and other synaptic vesicle proteins
Lymphoreticular antigens
Tetanus toxin binding sites
Neural cell adhesion molecules

both opened or closed types, and occur by themselves or in small aggregates, the so-called neuroepithelial bodies [4]. Neuroepithelial bodies are particularly conspicuous in the fetal lung and are located primarily at branch points of the bronchioles. The number of NE cells, which are basally located and have numerous dense core neurosecretory granules 100–120 nm in diameter, reaches a peak by wk 16–30 of gestation and decreases at about 6 mo of age. Lung NE cells produce a variety of peptides, such as serotonin, bombesin/gastric releasing peptide (GRP), calcitonin, and the recently identified ghrelin [5], although in hyperplastic or neoplastic conditions they may also produce adrenocorticotropic hormone (ACTH), vasoactive intestinal peptide (VIP), or somatostatin.

An NE component in the lung is also prominent in hypoxic conditions (i.e., high altitudes or chronic pulmonary diseases). NE cell functions are still to be completely understood, but GRP-containing cells have been found to be increased in infants with pulmonary dysplasia, cystic fibrosis, or prolonged assisted ventilation [6], thus suggesting their role in lung growth, development, and repair.

NE cell hyperplasia is an incidental microscopic finding, usually lacking clinical significance. There are three patterns that include (1) increased number of scattered NE cells, (2) linear proliferations along the bronchial mucosa, and (3) nodular hyperplasia consisting of increased number of NE bodies. While these proliferations can be related mainly to chronic bronchial inflammation [7], Langerhan's cell hystiocytosis [8], or bronchopulmonary dysplasia [9], a clinicopathologic syndrome called diffuse idiopathic pulmonary NE cell hyperplasia is typically associated with obliterative bronchiolar fibrosis in the absence of underlying conditions causing interstitial or airway fibrosis or inflammation.

At the extreme of this spectrum, the term tumorlets defines NE cell proliferations that extend beyond the subepithelial basal membrane, having a size <5 mm, and a dense fibrous stroma surrounding cell clusters.

The relationship between NE cell hyperplastic conditions and NE lung tumors is controversial. A common association between carcinoid tumors and both NE cell hyperplasia and tumorlets has been described [4,10]. On the other hand, the pathogenetic relationship between high-grade NE tumors, such as small cell and large cell carcinomas, and hyperplastic NE proliferations seems to be less convincing.

WELL-DIFFERENTIATED NE TUMOR—TYPICAL CARCINOID Typical carcinoid represents <1% of lung tumors. As opposed to other NE lung tumors, it is not related to smoking, and shows a mean age of 55 yr, with an equal male/female distribution. The most common location is in the proximal airways, presenting as a polypoid mass that protrudes into the bronchial lumen. Several histomorphologic patterns can be recognized, most often in combination. Tumor cells are polygonal, large, with abundant, finely granular and eosinophilic cytoplasm. Nuclei show clumped open chromatin and small dark nucleoli. The growth pattern most often resembles trabecular architecture with cords or ribbons of tumor cells dispersed in a delicate fibrovascular stroma and forming perivascular or true rosettes (Fig. 1A). Morphological pattern variants include spindle-cell type; acinar or glandular formations, even with mucin production; oncocytic changes; and clear cell features. Very rarely, they may contain melanin.

Necrosis is always absent and there are fewer than two mitoses per 10 HPF (corresponding to 2 mm^2). These features are the most important to differentiate typical carcinoids from other NE lung tumors of higher grade. The main differential pathological findings of NE lung tumors are summarized in Table 2, and are discussed in specific sections. In addition, a general scheme of immunohistochemical markers useful in the differential diagnosis of NE lung tumors is presented in Table 3.

Owing to their variable morphological growth patterns, typical carcinoids also enter into the differential diagnosis with other non-NE entities, especially in lung biopsy specimens.

Spindle-cell carcinoids should be distinguished from nerve sheath proliferations and hemangiopericytomas, the discriminating feature being the absence in typical carcinoid of glial fibrillary acid protein and of vascular markers, which are positive in nerve sheath tumors and hemangiopericytomas, respectively, in contrast to the presence of NE markers. Tumors with glandular formation could be difficult to distinguish from adenocarcinomas, especially when mucin secretion is observed. Clear cell changes could resemple clear cell (sugar) tumors, which are invariabily positive for CD34 and HMB45 and negative for NE markers.

The most common genetic feature in both typical and atypical carcinoids is the allelic deletion of the long arm of chromosome 11. Different loci in 11q are altered in these tumors, with 11q13 the most important, as it is linked to the *MEN1* gene locus, and is found to be lost in nearly one-third of sporadic carcinoids, both typical and atypical. The occurrence of lung carcinoids in familial multiple endocrine neoplasia type 1 (MEN 1) syndrome is rare, and lung carcinoids represent infrequently tumor entities in MEN 1 affected patients. *MEN1* gene alterations are usually absent in high-grade NE carcinomas, and *MEN1* kindreds do not develop these types of tumors as inherited carcinomas. Interestingly, familial lung carcinoid tumor syndromes different from MEN 1 have been described, and need to be better clarified.

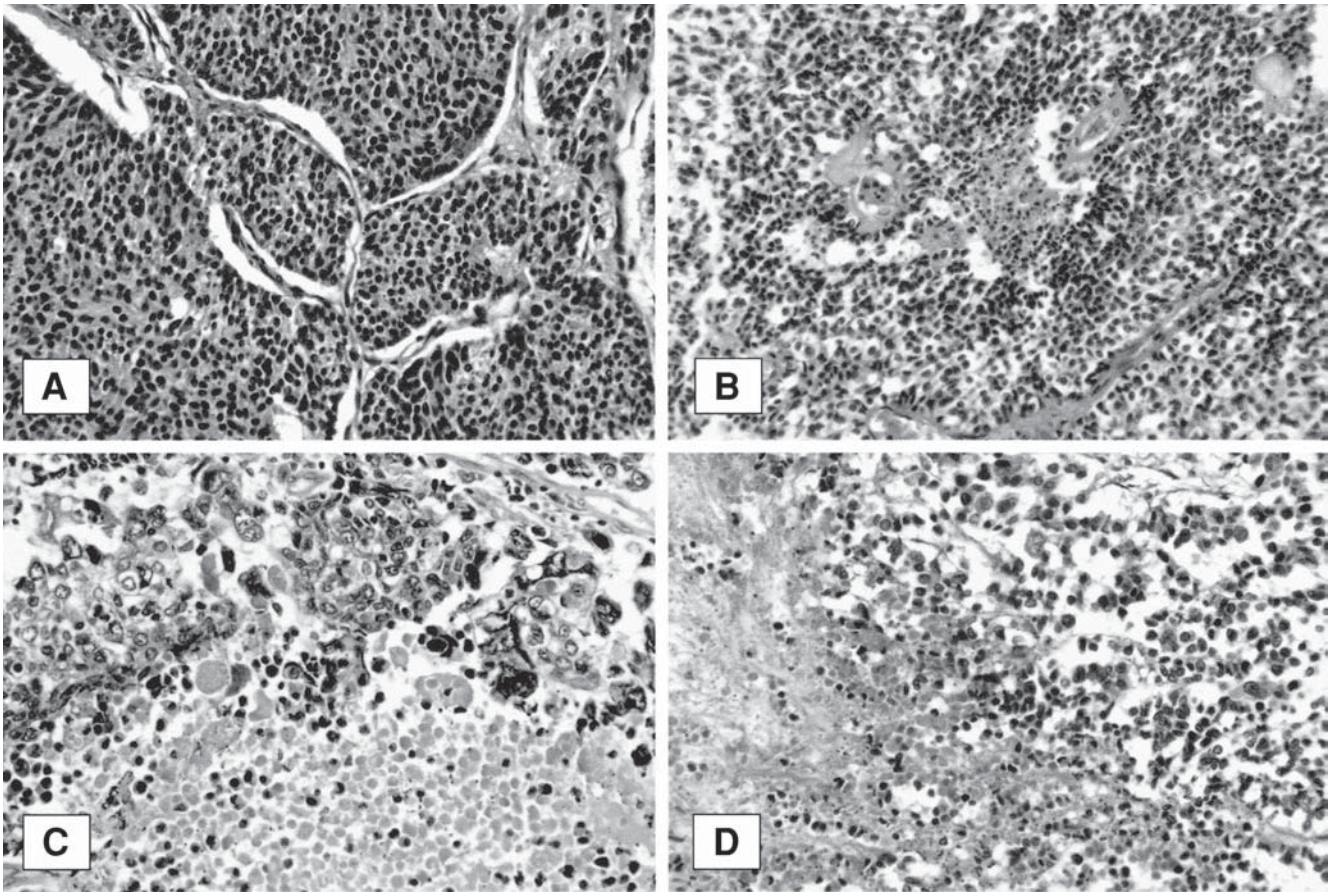

Figure 1 Morphologic features in neuroendocrine tumors of the lung. Typical carcinoid (**A**) shows characteristic organoid growth pattern, without cytologic atypia and necrosis. Punctate necrosis is a main feature of atypical carcinoid (**B**), and is clearly distinguishable from large infarctlike necrosis in large cell neuroendocrine (**C**) and small cell (**D**) carcinomas, the latter two having high mitotic activity. **A–D**: H&E, original magnification ×200.

Table 2
Pathologic Differential Findings in NE Lung Tumors

	Typical carcinoid	*Atypical carcinoid*	*Large cell NE carcinoma*	*Small cell carcinoma*
Organoid pattern	Characteristic	Characteristic	Present, less extensive	Absent
Cell size	Large	Large	Large	Small (less than the diameter of three lymphocytes
Cytoplasm	Abundant	Abundant	Abundant	Scant
Nuclear pleomorphism	Usually absent	Often present	Present	Present
Nucleoli	Small or absent	Small or absent	Present, often prominent	Absent
Mitosis	<2 × 10 HPF	2–10 × 10 HPF	≥11 × 10 HPF (mean 70)	Mean 70 × 10 HPF
Necrosis	Absent	Present, focal, or punctate	Present, large zones	Present, large zones
Disease-free survival at 5 yr	100%	69%	27%	9%

WELL-DIFFERENTIATED NE CARCINOMA—ATYPICAL CARCINOID Atypical carcinoids represent nearly 10% of endocrine lung tumors, even if it is difficult to compare different series because in some of them, large cell NE carcinomas are included in this same group. Atypical carcinoids are more often associated with cigaret smoking, have a male predominance, and are more frequently peripheral as compared to typical carcinoids. Conversely, they share the same morphologic, cytologic, and architectural features as typical carcinoids and the correct diagnosis of the two forms may be difficult, especially in small biopsies. As previously mentioned, the main differential pathologic features between the two forms are the presence of necrosis in atypical carcinoids and their higher (2–10 per 10 HPF) mitotic count. Necrosis has a peculiar focal, punctate, sharply demarcated appearance (Fig. 1B). Focal nuclear pleomorphism may also occur. Up to 20% of atypical carcinoids may lack NE markers. Peripherally located tumors more frequently show a spindle-cell appearance and have to be separated from small cell carcinomas on the basis of their architecture. The mitotic index (more than 20 mitoses per 10 HPF in small

Table 3
Immunohistochemical Markers in the Differential Diagnosis of NE Tumors of the Lung

	CgA, SNP	*HMW-CK*	*CK7*	*TTF-1*	*CLA*	*Ki-67*
Typical carcinoid	+	–	+	–	–	Very low
Atypical carcinoid	+	–	+	–	–	Low
Small cell carcinoma	+[a]	–	+	+	–	High
Large cell NE ca.	+[a]	–	+	+	–	High
Large cell non-NE ca.	–	+	+	–	–	Intermediate
Non-NE ca. with NE diff.	+ (Focal)	+	+	–	–	Intermediate
PD squamous carcinoma	–	+	+	–	–	Intermediate
PD adenocarcinoma	–	+	+	+	–	Intermediate
Basaloid carcinoma	–	+	+	–	–	Intermediate
Metastatic endocrine ca.	+	–	–/+	–	–	Variable
Lymphoma	–	–	–	–	+	Variable

CgA, Chromogranin A; SNP, synaptophysin; HMW-CK, high-molecular-weight-cytokeratin; CK7, cytokeratin 7; TTF1, thyroid transcription factor 1; CLA, common leukocyte antigen; NE, NE; ca., carcinoma; diff., diferentiated; PD, poorly differentiated.
[a]With prior heat antigen retrieval for CgA.

cell carcinomas) and the extent of necrosis should be considered first. The nuclear to cytoplasmic ratio, which is much higher in small cell carcinoma, and the nuclear chromatin, which is more dense and uniform in small cell carcinoma, may also be helpful features.

The distinction between atypical carcinoids and large cell NE carcinomas is even more difficult, and is discussed below.

LARGE CELL NE CARCINOMA Large cell NE carcinomas are defined as large cell carcinomas with organoid and trabecular growth patterns, showing NE differentiation and in which the latter can be confirmed by immunohistochemistry or electron microscopy. The main differential diagnostic problem is represented by atypical carcinoids, with which they may be easily confused. Large cell NE carcinomas present classical NE morphologic features, but to a lower extent as compared with atypical carcinoids, and may also grow in solid or lobular fashion. The type of necrosis, with a prominent infarctlike appearance in large cell NE carcinoma, and mitotic count (more than 11 per 10 HPF) help to differentiate the two forms (Fig. 1C). Nuclear features, such as larger nuclei with prominent nucleoli, higher pleomorphism, and a lower nuclear to cytoplamic ratio are also characteristic.

Large cell size distinguishes large cell NE from small cell carcinoma, but seems to be equivocal, even considering the possible occurrence of coexisting small and large cell components in the same tumor. Abundant cytoplasm, a more vesicular and dispersed chromatin, and the presence of prominent nucleoli favor the diagnosis of large cell NE carcinoma. Two other highly malignant tumors that enter into the differential diagnosis are basaloid and large cell non-NE carcinomas, but the absence of an organoid pattern of growth and of the previously described cytological features in both, as well as of NE markers, help to discriminate these two entities.

Diagnostic ICC features are positivity, at the cell membrane level, for neural cell adhesion molecute (N-CAM) [11] and focal positivity for CgA on the basis of antigen retrieval procedures.

SMALL CELL CARCINOMA Small cell carcinoma usually arises as a rapidly growing tumor of the major airways. It is made up of small cells with scant cytoplasm and condensed small nuclei, usually less than three lymphocytes in size. Neoplastic cells grow in diffuse sheets loosely connected in a minimal stroma. Pseudo-perivascular rosettes may occur. The distinction in three groups, according to the cell size, as oat cell, intermediate, and combined cell type, has a poor correspondence to clinical features and seems, at least for the first two forms, to be related more to tissue preservation, especially in biopsy specimens, than to true cell characteristcs. High mitotic rate and large infarctlike necrosis are the main features (Fig. 1D).

Small cell carcinoma should be differentiated from several small cell proliferations, both of epithelial and mesenchymal origin. With regard to the latter, pulmonary lymphomas may resemble small cell carcinoma, and several nuclear and cytologic features may overlap, making immunohistochemistry useful for differential diagnosis. Other small cell tumors that may be confused with small cell carcinoma include basaloid carcinoma, poorly differentiated squamous cell and adenocarcinomas, and peripheral neuroectodermal tumors of the thoracic wall (so-called Askin tumors). The differential diagnosis with the former three is made on the basis of immunohistochemical findings and on few morphologic features, such as loose connective tissue, nuclear condensation, and scanty cytoplasm, which are more prominent in small cell carcinoma. The discrimination with Askin tumor may be more difficult, owing to its positivity with NE markers and the diffuse growth fashion, with pseudo- and true rosette formation. In Askin tumor, however, the nuclei present a more dispersed chromatin, one or more small nucleoli, and specific immunohistochemical markers, such as MIC2 (CD99), are constitutively expressed.

Thyroid transcription factor-1 (TTF1) is positive in the majority of both large cell and small cell carcinoma cases; thus, it is useful to differentiate these lesions from high-grade NE carcinomas of other sites, but it is absent in both typical and atypical carcinoids [12]. NE lung tumors are negative with high-molecular-weight cytokeratins (such as 34βE12), in contrast with non-NE lung tumors [13].

The vast majority of small cell carcinomas, as well as most other lung carcinomas, show deletions of the short arm of chromosome 3, which are putative tumor suppressor genes still to

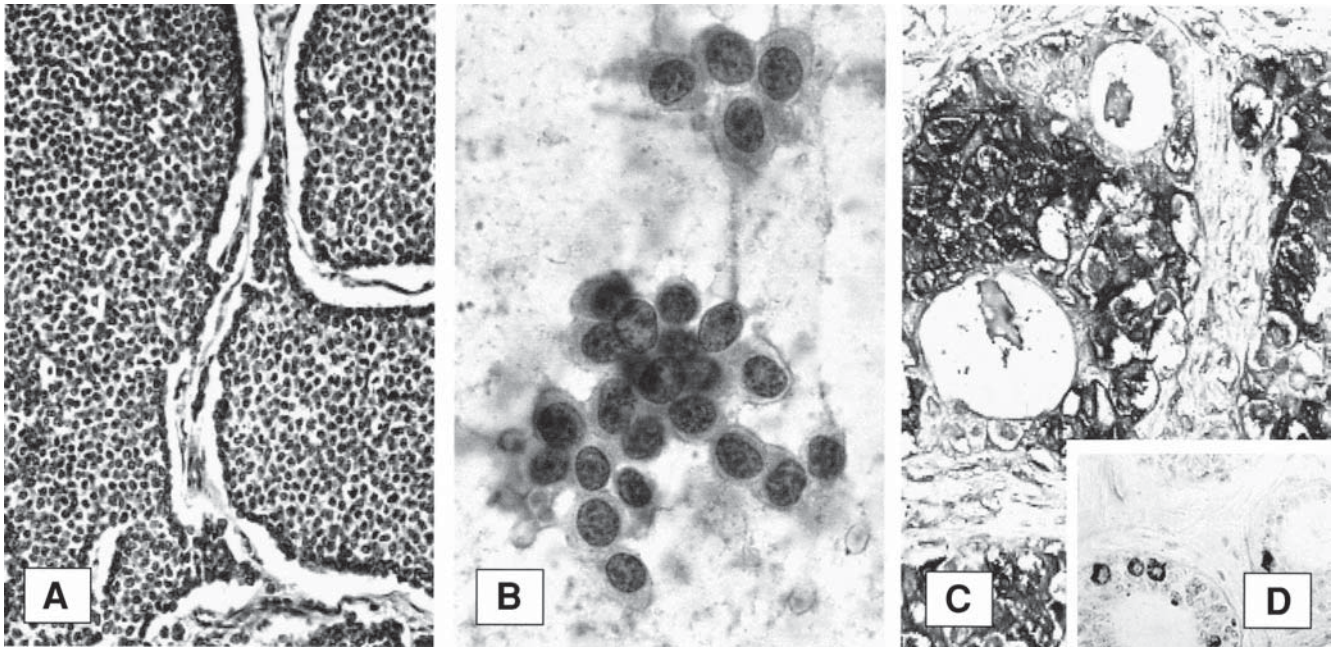

Figure 2 Endocrine breast carcinoma. Monomorphic cells grow in solid chords (**A**) and present a cytologic plasmocytoid appearance (**B**) with round nuclei showing mild atypia. Neoplastic cells are strongly positive for CgA, as compared to negative ductal structures (**C**). Peritumoral ducts contain single normal endocrine cells, as detected by CgA immunohistochemistry (**D**). **A**: H&E, ×200; **B**: H&E, ×800; **C**: immunoperoxidase, ×400; **D**: immunoperoxidase, ×200 (original magnification).

be completely identified. The von Hippel–Lindau (*3p25*) and FHIT (*3p14.2*) genes seem to be the most promising, and alterations of both, namely, loss of heterozygosity, have been detected in most cases of small cell carcinomas. With regard to cell cycle regulators, the *p53* gene is highly mutated in high-grade NE tumors, while normal in all typical and most atypical carcinoids. In this context, immunohistochemical detection of *p53* has been proposed in the differential diagnosis of NE lung tumors. Loss of function of retinoblastoma protein has also been detected in most small cell carcinomas.

COMBINED NE CARCINOMAS A small proportion of NE carcinomas are histologically heterogeneous. This occurs mostly with high-grade carcinomas, both small and large cell types, which may be in association with squamous cell carcinomas and adenocarcinomas, or, less frequently, with sarcomatous tumors. Combined carcinomas share many clinical, epidemiologic, and prognostic features of the NE counterpart, so that they are generally classified as variants of small and large cell NE carcinomas.

NONENDOCRINE CARCINOMAS WITH FOCAL NE DIFFERENTIATION As for many other organs, immunohisto-chemical recognition of the NE phenotype in the absence of light microscopical or ultrastructural findings of NE origin may be recognized in a subset of lung carcinomas, most frequently adenocarcinomas, blastomas, squamous cell carcinomas, and large cell carcinomas [14–17]. The percentage of immunohistochemically NE-positive cells may range from 10% to 20%. Little is known about the significance of NE phenotype of these tumors, and controversies are raised in the literature about their behavior and response to chemotherapy.

BREAST

NE CELLS IN THE NORMAL BREAST The presence of argyrophilic and CgA-reactive cells, located between the basal myoepithelial and the luminal epithelial cells, occasionally has been demonstrated in histologically normal breast tissue surrounding infiltrating endocrine breast carcinomas [18–21] (Fig. 2). However, nothing is known regarding specific hormonal peptides produced by breast tissue.

NE DIFFERENTIATION IN CARCINOMAS OFTHE BREAST

Definition Reports on breast tumors showing features similar to carcinoids date back to 1963 [22]. We recently defined as "endocrine differentiated breast carcinomas" a subset of tumors whose morphology and histochemical and immunocytochemical features overlap those of endocrine carcinomas of the gastrointestinal tract, lung, prostate, and other organs [23]. Following a quantitative approach, endocrine breast cancer are tumors that express NE markers in >50% of their cell population [23]. Only 2–5% of breast carcinomas fall within this specific definition; however, the percentage increases in the sixth or seventh decades of life [24]. The ability to produce specific endocrine markers easily detectable in the serum is of important clinical significance for follow-up of patients.

Cytology Cytological features of endocrine carcinomas are very peculiar and useful for diagnosis [25]. The endocrine cells are plasmacytoid, spindle shaped, or with a signet ring appearance and show intracytoplasmic granules particularly evident with Giemsa and Diff-Quick stains. Dark hyperchromatic nuclei, "crush artifacts," and nuclear streaming are conversely the hallmarks of small cell carcinoma on cytological samples.

Histotypes Endocrine ductal *in situ* carcinoma (DCIS) is recognized because granulated plasmacytoid or spindle cells grow within ducts featuring solid sheets and festoons lining delicate fibrovascular septa. Mucin production may be detected within the neoplastic ducts [21]. Small cell DCIS has also been described.

Solid-cohesive carcinoma or low-grade insular carcinoma is the morphological variety more similar to carcinoid tumors. In fact, this infiltrating carcinoma is formed by highly cellular nests, or trabeculae of endocrine cells, with peripheral cell palisading, reminiscent of carcinoid tumors [23]. Rarely, the cells are organized in glandular or rosettelike structures. Endocrine breast carcinomas may also mimic atypical carcinoid tumors of the lung showing whirls of spindle cells, with low nuclear pleomorphism, higher mitotic count, and focal necrosis.

In other cases the endocrine cells are large, clear, and granulated and organized in round alveolarlike structures, separated by scanty dense stroma that produce an infiltrative growth pattern similar to the alveolar variant of lobular carcinoma [23].

Mucinous differentiation is observed in 26% of endocrine carcinomas of the breast [24]. In endocrine mucinous carcinoma, quite large cribriform or solid islands of plasmacytoid or spindle cells float within mucin lakes, which stain with periodic acid-Schiff (PAS) and alcian blue. Maluf and Koerner [26] adopted the descriptive term "cellular mucinous carcinoma" to differentiate the endocrine variant of mucinous carcinoma from the nonendocrine one. In the so-called "solid papillary carcinoma," solid round nests of cells with papillary features intermingle with small lakes of extracellular mucin [27].

Following the classical criteria of grading, all the above-described varieties of endocrine carcinomas are well- or moderately differentiated tumors expressing estrogen and progesterone receptors. About 50% of such tumors express CgA and CgB and only 16% express synaptophysin [24].

Poorly differentiated endocrine carcinoma represent 15% of endocrine breast carcinomas. The diagnosis of poorly differentiated endocrine carcinoma is suggested by the extremely high number of mitoses, ranging from 18 to 65 per 10 HPF, in a tumor that maintains the endocrine architecture and expresses CgA [24].

Finally, small cell/oat cell carcinomas of the breast need to be kept distinct from undifferentiated carcinomas. These are morphologically indistinguishable from their counterparts in the lung; however, in primary breast small cell carcinomas an *in situ* component with the same cytological features may be present. Neuron-specific enolase (NSE) is expressed in 100% of small cell carcinomas of the breast [27], whereas CgA and synaptophysin are expressed in about 50% of such cases. In addition, 20% of small cell mammary carcinomas express TTF1 [28]. Primary mammary small cell carcinomas are cytokeratin 7-positive and cytokeratin 20-negative, whereas, for example, lung small cell carcinomas are negative for both [27]. In addition, estrogen receptors are expressed in more than 50% of small cell carcinomas [27].

However, immunodetection of panendocrine markers may fail to recognize endocrine tumors, which produce but do not retain the specific antigen in the cells. When the morphological suspect of the endocrine nature of a tumor does not correlate with the immunophenotype, the absence of the expression of the high-molecular-weight cytokeratin (clone 34βE12), which selectively labels non-NE carcinomas, may support the endocrine tumor diagnosis and induce different types of analysis [29].

DIVERGENT DIFFERENTIATION A relatively common phenomenon in endocrine breast carcinomas is the presence of a "divergent differentiation," which indicates the ability of a tumor to produce both exo- and endocrine substances. Mucin production is indeed a common feature in well-differentiated endocrine breast carcinomas. Recently, we have demonstrated that apocrine differentiation is present in about 50% of well and moderately differentiated endocrine breast carcinomas as well [24], while poorly differentiated carcinomas do not show this multidifferentiation capacity and express the endocrine markers, only.

The immunocytochemical and *in situ* hybridization coexpression of endocrine and apocrine (gross cystic disease fluid protein [GCDFP]-15) markers in tumors with identical morphological substrate may lead to the hypothesis of an uncommitted stem cell capable to differentiate toward both endocrine and apocrine lineage. Interestingly, expression of apocrine differentiation markers in endocrine breast carcinomas is typical of elderly women and androgen receptors are expressed by 80% of cells of endocrine–apocrine differentiated tumors [24]. It can be speculated that the relative prevalence of androgenic hormones in the menopausal period may induce the production of apocrine proteins through the activation of specific androgen receptors.

CLINICOPROGNOSTIC PARAMETERS A major concern stems from the impact on the patient's clinical history and prognosis when a diagnosis of endocrine carcinomas of the breast is made. Although the histological patterns described above are useful to diagnose an endocrine carcinoma correctly, production and secretion of specific markers and the histological grade of differentiation are the two factors with major clinical and prognostic impact.

Implicit in the definition of endocrine breast carcinomas as "tumors that express endocrine markers in more than 50% of their cells" is the possibility of taking into account the serum levels of chromogranins or NSE in the patient's follow-up.

Regarding the clinical evolution of endocrine breast carcinomas, we demonstrated that similar to conventional breast carcinomas, the histological grade is one of the most important parameters to predict clinical evolution [29]. Poorly differentiated endocrine carcinomas, which showed a high proliferative activity, are very aggressive. On the other hand, well-differentiated tumors with low proliferative activity could be considered as benign, with all patients surviving at long-term follow-up.

Mucin production is correlated with a better prognosis; however, the grade overcame the importance of such differentiation to predict the clinical evolution. In addition, the association with apocrine differentiation seemed to improve long-term survival of patients with endocrine breast tumors [24].

NONENDOCRINE CARCINOMAS WITH FOCAL NE DIFFERENTIATION Carcinomas not otherwise specified (NOS), which show immunocytochemical expression of NE markers by scattered cells [30], are excluded from the subset of endocrine breast carcinomas. Focal expression of NE markers in

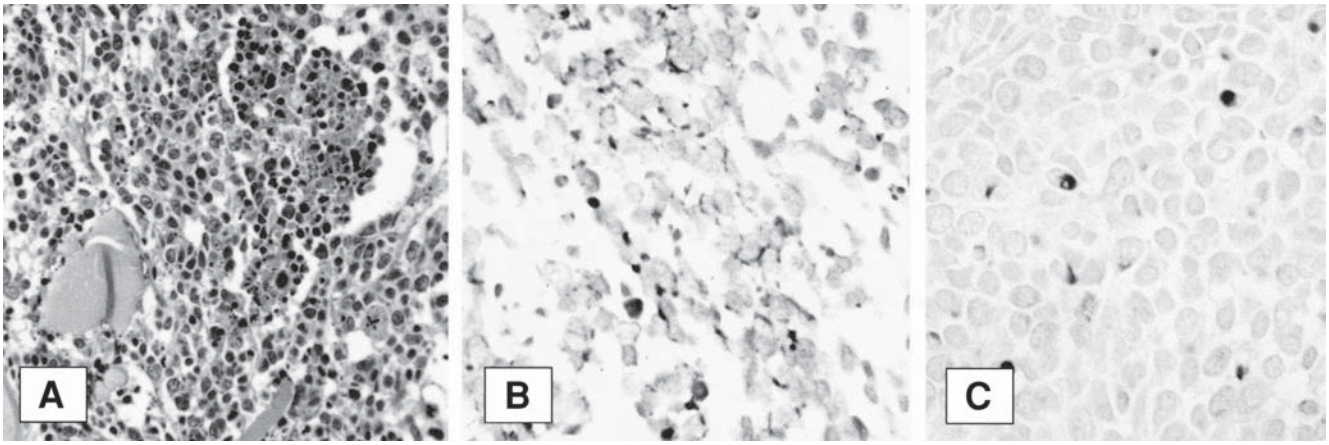

Figure 3 Merkel cell carcinoma. Small-sized neoplastic cells with marked atypia, focal necrosis, and high mitotic activity, grow in a diffuse fashion (**A**) and express both epithelial (pancytokeratins in peculiar dotlike appearance) (**B**) and neuronal (neurofilaments) (**C**) markers. **A**: H&E, ×200; **B**: immunoperoxidase, ×200; **C**: immunoperoxidase, ×400 (original magnification).

breast carcinomas is in line with similar observations in other exocrine tumors of endodermal (lung, gastrointestinal tract) [31,32] and ectodermal (skin) [33] origin and does not carry any clinical or prognostic significance.

SKIN

NE CELLS IN NORMAL SKIN AND IN NON-NEOPLASTIC CONDITIONS In 1875 Frederick Sigmund Merkel described a cell located singly in the basal layer of the epidermis and in proximity of hair follicles and other adnexa [34]. It was found in many species, including mammals and humans. The oral mucosa and lips also contain scattered Merkel cells [35]. The Merkel cell function is that of a mechanoreceptor. The cells are associated with nerve fibers and are numerous in the epithelium of the lips and fingers as well as in areas with high hair density. Merkels cell have been found to produce several hormonal peptides, including VIP and bombesin [35]. The immunophenotypic profile of Merkel cells includes expression of panendocrine markers, namely, CgA, NSE, and synaptophysin (but not neurofilaments) and the presence of low-molecular-weight cytokeratin (CK), such as CK 18, 19, and 20. Neurosecretory granules measuring 80–200 nm were found on electron microscopy examination. Hyperplasia of NE cells of the skin in response to chronic injury (e.g., radiation dermatitis) was also described in analogy with the "linear" type of hyperplasia identified in the bronchial and gastric mucosa [35].

MERKEL CELL CARCINOMA Merkel cell carcinoma is a very rare skin tumor, described 30 yr ago [36] as a variety of sweat gland carcinoma with prominent trabecular features. It was subsequently found to be of NE origin and related to normal Merkel cells of the skin, based on ultrastructural and phenotypic similarity with such cells.

Synonyms This tumor is reported under several designations, including: trabecular carcinoma, endocrine carcinoma of the skin, NE carcinoma of the skin, Merkel cell tumor, primary small cell carcinoma of the skin (PSCCS), cutaneous NE carcinoma, and Merkel cell carcinoma.

Clinical Data The exact incidence of this very rare tumor is not known. More than 800 cases are on record in the literature [37]. It affects the elderly population (>65 yr old) with no gender preference. The classical location is the head and neck area (up to 65% of cases in some series). The other cases are almost equally distributed between limbs and trunk [38]. In individual cases an association with other skin diseases (squamous or basal cell carcinomas, Bowen's disease, actinic keratosis) or with systemic disease (chronic lymphocytic leukemia, polycythemia vera, chronic renal failure, and—in general—with immunosuppression) have been reported [39].

Gross Features Merkel cell carcinoma appears as a small dermal nodule or as a plaque of approx 2 cm that may ulcerate the overlying skin or discolor it. Multiple small satellite nodules around the primary tumors have also been described.

Microscopic Features The classical appearance is that of a highly cellular dermal tumor mass extended to the subcutaneous fat or to the epidermis, which can be ulcerated. The classical pattern is that of a diffuse cohesive growth of medium-size or small cells (Fig. 3A), but several other patterns of growth have been observed in Merkel cell carcinoma, including the originally described trabecular pattern (the least common, actually) and a diffuse noncohesive pattern in which tumor cells are dissociated from each other and strongly resemble a lymphoma or leukemia. The tumor cells are rather uniform, round to ovoid with centrally located nucleus and a very thin rim of cytoplasm. Chromatin is finely dispersed and dusty and nucleoli are rarely prominent. Mitoses are numerous. DNA encrustations (Azzopardi's phenomenon) have been observed only occasionally in Merkel cell carcinoma and, if extensively present, it is better to orient the diagnosis toward a small cell lung carcinoma metastasis. Necrosis and vascular invasion are common features. The stroma is usually scant and vascular, and contains several small lymphocytes that need to be distinguished from small tumor cells, especially in the evaluation of surgical margins of frozen sections [39]. Merkel cell carcinoma may be associated with other lesions [40]. The most commonly encountered is *in situ* or invasive squamous cell carcinoma, either in the primary Merkel

Table 4
Differential Diagnosis of Merkel Cell Carcinoma

Parameter	*Merkel cell ca.*	*Basal cell ca.*	*Small cell ca.*	*Lymphoma*
Tumor location	Dermis	Epidermis	All thickness	Epidermis/dermis
Growth pattern	Trabecular/solid	Nests/chords/solid	Diffuse	Diffuse/nodular
Necrosis	+	+/–	++	–
Vascular invasion	++	+	+	+
Cell size	Small	Small/medium	Small	Small/large
Cytoplasm	Scant/amphophilic	Eosinophilic	Scant	Scant
Chromatin	Dusty	Condensed	Condensed	Condensed
Nucleoli	Incospicuous	Small	Absent	Small
DNA encrustations	–	–	+	–
Mitotic count	Variable	Variable	High	High
LMW cytokeratin	+ (Dotlike)	+	+	–
HMW cytokeratin	–	+	–	–
Endocrine markers	+	–	+	–
Lymphoid markers	–	–	–	+

Ca, Carcinoma; LMW, low molecular weight; HMW, high molecular weight.

cell carcinoma or in its lymph node metastases. More rarely described are glandular differentiation or a lymphoepitheliomalike carcinoma component in a Merkel cell carcinoma [41] or a sarcomatoid component in an otherwise classical Merkel cell carcinoma in which pleomorphic eosinophilic desmin-positive, myogenin-positive cells were found intermingled with the small Merkel tumor cells [33].

Immunophenotype Epithelial markers are present in Merkel cell carcinoma, including epithelial membrane antigen (EMA) and cytokeratins. Low-molecular-weight cytokeratins, such as cytokeratin 20, are typically expressed by Merkel cell carcinoma and the immunostaining provides either a diffuse cytoplasmic pattern or—more commonly—a dotlike paranuclear reaction (Fig. 3B). High-molecular-weight cytokeratin (e.g., cytokeratins 1, 5, 10, 14 recognized by monoclonal 34BE12) are usually absent in Merkel cell carcinoma. The opposite pattern (presence of high-molecular-weight and absence of low-molecular-weight cytokeratins) is recognized in nonendocrine skin carcinoma. Panendocrine markers are variably (20–100%) expressed in Merkel cell carcinoma, including NSE, neurofilament proteins (Fig. 3C), synaptophysin, and CgA. Finally, reactivity for several different hormones has been reported, including ACTH, calcitonin, somatostatin, VIP, bombesin, and others [40]. Cytogenetic studies have shown that deletions and unbalanced translocations may involve the short arm of chromosome 1 in up to 40% of the investigated cases [42]. No data have been reported on tumor cell proliferation or oncogene expression in Merkel cell carcinoma. In our own series of more than 50 cases, a wide range of proliferative activity as detected by Ki-67 immunostaining was found, which was not able to discriminate clinically aggressive from indolent tumors (M. Papotti and M. Volante, *unpublished observations*).

Behavior Merkel cell carcinoma is a highly invasive tumor, which frequently infiltrates surrounding tissues and organs. If a wide surgical excision with at least 3 cm of free margins has been performed, local recurrences are uncommon. Locoregional lymph node metastases are relatively frequent and distant metastases (to lung, bone, and liver) are reported in up to one third of cases. The 5-yr survival rate is between 30% and 65%, according to different reported series. The mortality was found to be strikingly different in patients who developed distant metastases (63% in females and 85% in males) as compared to that of patients without distant metastases (4%) [43]. No reliable prognostic parameters have been identified so far, except for the presence of distant metastases. Tumor size and location were claimed to be of discriminatory value, but this was not confirmed in subsequent studies. The treatment of choice is surgical, with wide excision of at least 3-cm margins and dissection of palpable regional nodes. Radiation therapy follows. Advanced cases do not gain any benefit from radio-or chemotherapy. Hormonal treatment with somatostatin analogs has also been reported in a few cases [44], based on the expression of somatostatin receptors in Merkel cell carcinoma [45,46].

Differential Diagnosis Merkel cell carcinoma may share several morphological similarities with small cell carcinoma of the lung and other locations. Dotlike cytokeratin immunostaining was regarded as a specific differential marker, but some authors found this same pattern in up to 35% of small cell lung carcinomas [47]. The combined evaluation of a panel of markers, which include cytokeratin 20, neurofilament proteins, and TTF1 [48], will address to a correct diagnosis. The diffuse noncohesive variants of Merkel cell carcinoma has to be distinguished from non-Hodgkin's lymphomas. Morphologically, the finely dispersed chromatin in the absence of a prominent nucleolus usually points to a Merkel cell carcinoma. Immunohistochemistry is generally very helpful in distinguishing these different tumors (Table 4).

PRIMARY CARCINOIDS OF THE SKIN Primary carcinoids of the skin are exceedingly rare. Six cases are on record in the English literature to date [49–54]. All patients had a solitary dermal nodule, 1–4 cm in size, in the trunk or scalp.

Histologically, these cases had a typical carcinoid appearance, in the absence of any other known extracutaneous location. One case was a mucinous carcinoid [52] as classically observed

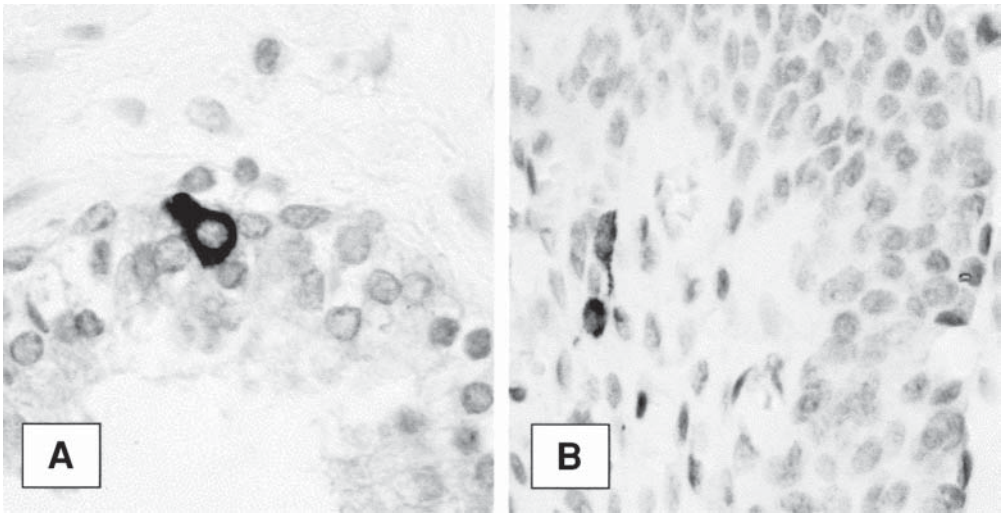

Figure 4 Normal neurodocrine cells in the prostate (**A**) and urinary bladder (**B**), both of the closed type, as detected by CgA immunohistochemistry. **A,B**: Immunoperoxidase, ×400, original magnification.

in the appendix. The others were insular or trabecular well-differentiated endocrine tumors.

The phenotype was that of typical carcinoids with panendocrine marker expression, argyrophilia, and the presence of neurosecretory granules at electron microscopy.

A favorable prognosis was reported in all cases. The origin of this tumor is debated. Although endocrine cells of the skin are well known, an alternative hypothesis is that at least some of these cases represent variants of basal cell carcinoma, which can show foci or NE differentiation and also areas of palisading and trabecular growth [51].

The morphological appearance overlapped that of the more common metastases of carcinoid tumors to the skin, and the final diagnosis is based largely on the exclusion of primary endocrine tumors in other locations.

NE DIFFERENTIATION IN NONENDOCRINE CARCINOMAS OF THE SKIN

Basal Cell Carcinoma The presence of endocrine cells in basal cell carcinoma was originally described in 1979 by Eusebi and co-workers [55], using argyrophilic methods and electron microscopic demonstration of dense-core neurosecretory granules. Subsequent reports demonstrated several NE products (both hormones and panendocrine markers) in basal cell carcinomas by means of immunohistochemistry and/or reverse transcriptase-polymerase chain reaction (RT-PCR) [56–59]. The percentage of endocrine differentiated basal cell carcinomas varies according to the detection method of NE markers. At present, such divergent differentiation does not seem to affect prognosis of basal cell carcinoma. The origin of the NE cell population in basal cell carcinoma is debated. It seems related to the appearance of an NE phenotype in otherwise classical basal cell carcinoma rather than to entrapment of normal Merkel cells of the skin, as no parallel cytokeratin 20 immunoreactivity (marker of Merkel cell carcinoma) was ever found in these tumors [33]. Mixed basal cell carcinoma and Merkel cell carcinoma was also reported [60].

Adnexal Carcinoma Both apocrine and mucinous eccrine carcinomas have been reported [40,61]. In the former, classical apocrine eosinophilic cells were admixed with a minor cell population showing argyrophilia and CgA expression. This endocrine cell population increased in recurrence and in metastatic deposits, suggesting a Merkel cell differentiation, although the expected cytokeratin 20 was never found in such cell components [40]. The latter tumor was a classical mucinous carcinoma as commonly found in breast and colorectal locations, with single chromogranin-reactive cells in the tumor cell nests dispersed in the mucinous lakes. It was not reported whether such cells had Merkel cell features [61], but the reverse condition, that is, a Merkel cell carcinoma with eccrine features, has been reported [62].

Follicular Differentiated Tumors Recent reports [63,64] indicated that trichoblastoma and trichofolliculomas contain endocrine cells with the phenotype of the Merkel cells. The latter are abundant in hair follicles of the fetal, but not of the adult, skin. Their occurrence in follicular-derived tumors may recapitulate the embryological developmental stages, and is probably related to a hyperplastic condition rather than to a neoplastic divergent cell population [64].

PROSTATE AND UROTHELIUM

NE CELLS IN PROSTATE GLAND AND UROTHELIUM

NE cells of the prostate include both opened and closed types with a predominance of the latter form. The majority of these cells present multiple dendritic processes with nervelike varicosities extending between adjacent epithelial cells and occasionally abutting on other NE cells (Fig. 4A). Ultrastructural analysis shows a wide diversity of neurosecretory-type granules containing different secretory products, including serotonin [65–67] and a variety of peptides, such as the chromogranins [68,69], the calcitonin family of peptides including calcitonin [70,71], a thyroid-stimulating hormonelike peptide [72], bombesin/gastrin releasing peptide [6,70], somatostatin

Table 5
Immunohistochemical Markers in the Differential Diagnosis of NE Tumors of the Prostate and Urinary Bladder

	CgA, SNP	*HMW-CK*	*PSA*	*AR*	*CLA*	*Ki-67*
NET/low grade NEC prostate	+	–	–	–	–	Low
High-grade NEC prostate	+[a]	–	–	–	–	High
Poorly differentiated ca. prostate	–	–	+	+	–	Intermediate
High-grade NEC bladder	+[a]	–	–	–	–	High
Poorly differentiated ca. bladder	–	+	–	–	–	Intermediate
Paraganglioma	+	–	–	–	–	Low
Lymphoma	–	–	–	–	+	Variable

CgA, Chromogranin A; SNP, synaptophysin; HMW-CK, high-molecular-weight cytokeratin; PSA, prostatic specific antigen; AR, androgen receptor; CLA, common leukocyte antigen; NET, NE tumor; NEC, NE carcinoma; ca., carcinoma.
[a]With prior heat antigen retrieval for CgA.

[73] and parathyroid hormone related protein [74,75]. NE cells lack expression of the androgen receptor [76,77].

It is likely that NE cells of the prostate regulate both cell growth and exocrine secretory activity through autocrine/paracrine circuits. In fact, the long dendritic processes suggest a paracrine regulation of adjacent epithelial cells, and there is evidence showing that such NE cell processes often make contact with each other. The presence of nerve processes in association with prostatic NE cells suggests that these cells may be active in neurocrine, in addition to paracrine and autocrine, regulation. NE cells in the prostate are more prominent in normal or atrophic prostate than in hyperplastic foci, and apparently are present in a larger amount in the transitional zone [78].

In the urinary bladder, NE cells were first described by Feyrter [79]. Fetissof [66] established that the endocrine cells in the urothelium were predominantly of the closed type, being the apex of the NE cell covered by the cytoplasm of adjacent epithelial cell (Fig. 4B). Little is known about their endocrine functions, but immunohistochemical studies demonstrated that they produce serotonin but lack peptide hormones such as ACTH, gastrin, glucagons, or somatostatin.

WELL-DIFFERENTIATED NE TUMORS (NET) AND CARCINOMAS (NEC) Well-differentiated NET (prostatic carcinoid) and NECs in the prostate are extremely rare, occurring isolated or combined with classic adenocarcinoma [80–82]. Similar tumors in the urinary bladder have not yet been reported.

Generally prostate-specific antigen (PSA) levels are not elevated. Rarely hormonal symptoms are present [83]. Histologically, NETs and NECs of the prostate show a carcinoidlike appearance characterized by solid, acinar, and cribriform structures formed by cuboidal and columnar cells. NECs, in contrast to NETs, show either vascular and/or perineural invasion or metastases to lymph nodes and distant organs [81]. The proliferation index, evaluated with MIB-1, is low and can help to differentiate these tumors from small foci of undifferentiated, malignant solid or trabecular carcinoma of the prostate (Table 5).

POORLY DIFFERENTIATED (SMALL CELL) NE CARCINOMAS Most NECs of the prostate and almost all NECs of the urinary bladder (first described by Cramer in 1981) [84] belong to the poorly differentiated or high-grade category. The frequency of high-grade NECs, when compared with all carcinomas of both organs, is very low, ranging from 0.2% to 1.0% [85–87]. The tumor usually affects patients older than 50 yr.

In prostatic tumors, as for well-differentiated tumors and carcinomas, PSA levels generally are not elevated. Cushing's syndrome due to production and secretion of ACTH can be associated [83]. Other paraneoplastic syndromes include myasthenic (Eaton–Lambert) syndrome [88], malignant hypercalcemia [89,90], and a syndrome of inappropriate antidiuretic hormone secretion [91].

On histological examination, small cells with hyperchromatic nuclei and extremely scanty cytoplasm are arranged in a predominantly solid fashion, sometimes associated with occasional "rosette" formation. The tumor cells form strands or clusters, either of oat cell or intermediate cell type, and have hyperchromatic nuclei that lack distinct nucleoli (Fig. 5). Occasionally the peripheral cells can show a palisading aspect. A large cell variant has also been described [92]. A variable degree of necrosis may be present. The growth pattern is diffusely invasive.

MIXED HIGH-GRADE NECS AND ADENOCARCINOMAS Prostatic small cell carcinomas can be associated with classic adenocarcinoma [85]. Moreover, an association with sarcomatoid and squamous carcinoma components can occur. In the urinary bladder mixed NE small cell and transitional cell carcinomas have been described.

NON-NE CARCINOMAS WITH FOCAL NE DIFFERENTIATION The occurrence of NE differentiation in non-NE tumors has been described almost exclusively in the prostate. Almost all adenocarcinomas of the prostate contain isolated NE cells. Focal NE differentiation is seen in virtually all prostate carcinomas, but is very uncommon in bladder cancer.

NE differentiation cannot usually be appreciated without special immunostainings, as peculiar morphologic changes are almost absent, except in those cases showing a Paneth cell-like change ("red cells" within the glands, containing large eosinophylic secretory granules) [93] (Fig. 6). This cell pattern is usually patchy, and present in acinar carcinoma as well as in cribriform areas. The nuclei of Paneth cell-like cells are vesicular with prominent nucleoli. Lysozyme immunoreactivity is invariably negative.

The NE tumor cells in adenocarcinomas seem to develop from atypical secretory tumor cells and are able to express hormones (serotonin, calcitonin, somatostatin) and prostate-specific antigen, in contrast to the cells of high-grade NECs [73,94].

These NE cells are negative for the proliferation marker MIB-1 [94], and they do not express the androgen receptor [77,

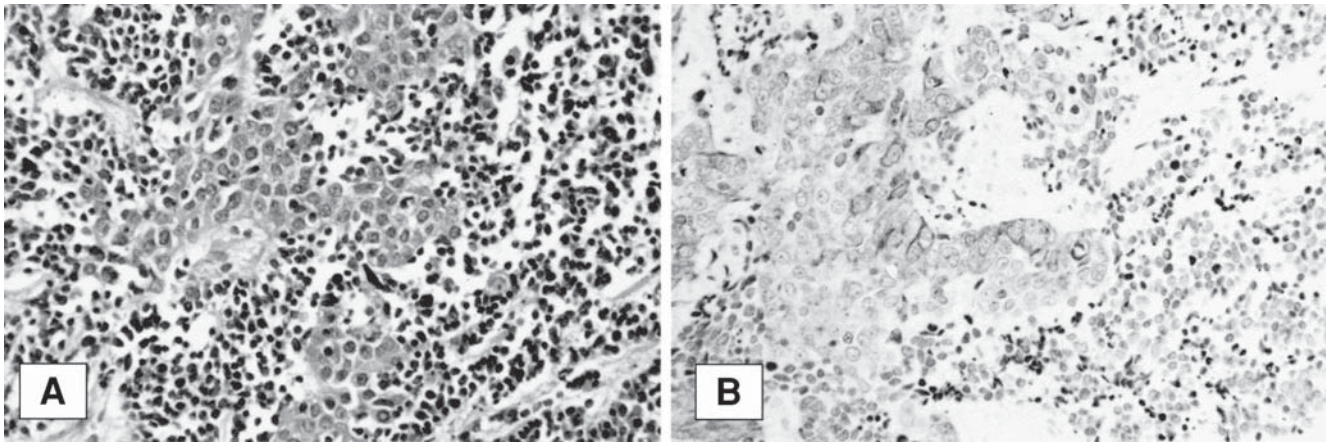

Figure 5 Poorly differentiated neuroendocrine carcinoma of the bladder. A mixed small and large cell neoplastic population is shown (**A**), lacking expression of high molecular weight (34βE12) cytokeratins (**B**). **A**: H&E, ×200; **B**: immunoperoxidase, ×200 (original magnification).

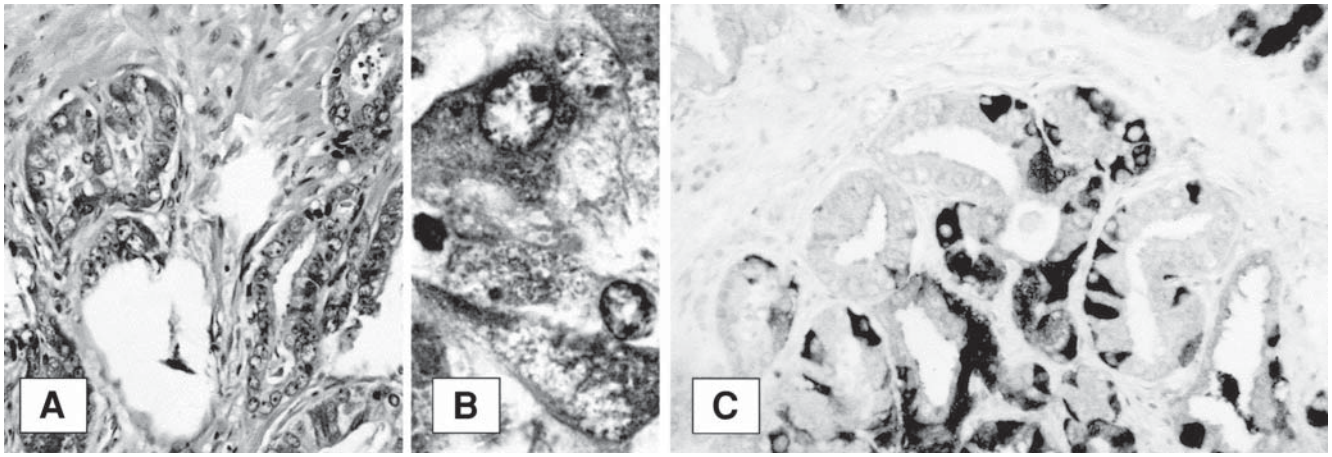

Figure 6 Adenocarcinoma of the prostate having neuroendocrine differentation features. Neoplastic glands contain large cells with abundant granular cytoplasm, having a characteristic Paneth-like appearance (**A,B**). These same cells strongly express CgA (**C**). **A**: H&E, ×200; **B**: immunoperoxidase, ×1000; **C**: immunoperoxidase, ×200 (original magnification).

94]. The prognostic significance of NE differentiation in conventional prostatic adenocarcinomas is still controversial. Several studies failed to demonstrate an influence on survival. Some data suggest that the extent of NE differentiation in the tumor at initial assessment is prognostically less important than in advanced prostate cancer, particularly in hormonal-therapy-resistant tumors. In fact, the absence of androgen receptor expression by NE cells could reflect a hormone-resistant phenotype in those tumors showing a high amount of NE cells, leading to a more rapid progression as compared to those cases having only occasional NE elements. Several investigators have found that there is an increase in NE differentiation in hormone-manipulation-resistant cancer with possible prognostic significance [95,96]. In contrast, Segawa and co-workers [97] found that the focal NE differentiation of prostate carcinoma is not a prognostic factor.

PARAGANGLIOMA Paraganglioma is a special NE tumor that occurs particularly in the wall of the urinary bladder [98]. It may originate from groups of paraganglionic cells present in the wall of the urinary bladder and in the outer regions of the prostate.

Paragangliomas are characterized by a typical "zellballen" growth pattern, as they are positive for conventional NE markers.

In the urinary bladder, these tumors may look like polyps that can be resected by simple excision. Paragangliomas are sometimes incidental findings after radical cystectomy. Presenting symptoms are hematuria and attacks of hypertension during micturition Multifocality is present in 20% of cases. They can metastasize to distant organs and to lymph nodes.

Paragangliomas of the prostate have only rarely been described [99] and seem to be predominantly malignant.

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19 Multiple Endocrine Neoplasia

TORU KAMEYA, MD, T. TSUKADA, MD, PHD,
H. FUTAMI, MD, PHD, AND K. YAMAGUCHI, MD

HISTORICAL OVERVIEW [1]

For the past 20 years or so, the multiple endocrine neoplasia (MEN) syndromes have attracted the interest of internists, endocrine surgeons, and pathologists, reflecting both the uniqueness of their clinical and pathologic presentations and the new discovery of important cellular mechanisms responsible for growth, development, and function of several lineages of hormone-secreting cells.

Reports dealing with these syndromes date back to the turn of the 20th century. Erdheim [2], in 1903, was the first to bring multiple endocrine hypersecretory states together into a single syndrome. He recognized a patient with a pituitary eosinophilic adenoma and parathyroid gland hyperplasia. In 1954, Wermer [3] identified a group of individuals who presented with adenomas of different endocrine glands in a distribution that would be at present defined as MEN type 1. Importantly, he recognized that the syndrome was heritable and appeared to be transmitted in an autosomal-dominant pattern. Sipple [4] in 1961 noted a linkage between pheochromocytoma and thyroid carcinomas. This was followed in 1968 by Steiner's classification [5] of the polyglandular hyperplasia syndromes into two groups, multiple endocrine adenomatosis types 1 and 2 (the nomenclature was later revised to multiple endocrine neoplasia) based on the type of endocrine gland involved. Then, Chong et al. [6] subdivided MEN 2 based on the presence of hyperparathyroidism (MEN 2A) or mucosal neuromas (MEN 2B). MEN syndromes are summarized in Table 1.

Recent developments, particularly those related to the molecular genetics of these syndromes, have given numerous important insights into their underlying pathogenesis. The present chapter offers a summary of pathological findings intimately associated with these syndromes and then focuses on more recent advances made in identification and analyses of the gene or genes that are responsible for their phenotypic manifestations.

OVERVIEW OF MEN 1 [7,8]

The term multiple endocrine neoplasia (MEN) encompasses genetically determined disorders with predisposition to hyperplastic and neoplastic lesions in two or more endocrine organs of the same patients [8]. MEN 1 is a disease with an estimated prevalence between 0.02 and 0.2 per 1000. It affects both sexes equally and has no geographic, racial, or ethnic preferences. The disorder may either be inherited as an autosomal-dominant trait or may occur sporadically as a result of new mutations.

It has a high degree of penetrance. Forty-three percent of patients who are carriers of the disease gene have clinical evidence of MEN 1 by the age of 20 yr, 85% by the age of 36 yr, and 94% by the age of 50 yr [9]. The endocrine tumors characterizing MEN 1 develop synchronously or metachronously. They involve the parathyroid, pancreas, duodenum, and anterior pituitary gland.

Less commonly, they also involve the thymus, lung, and gastrointestinal tract. Furthermore, adrenocortical, lipomatous, and skin lesions (angiofibromas, collagenomas, etc.), and ependymomas may also occur [9]. Clinical symptoms are most commonly caused by the lesions of the parathyroid (80–98%), the pancreas, and duodenum (40–85%), and the anterior pituitary (9–40%) [10].

In some patients, the clinical presentation is dominated by one manifestation. Evidence of MEN 1 is found in approx 18% of unselected patients with primary hyperparathyroidism, 15–40% of patients with Zollinger–Ellison syndrome (ZES), 4% of patients with insulinomas, 1.5% of patients with gastrointestinal neuroendocrine (carcinoid) tumors, and 2.7% of patients with pituitary adenomas. Therefore, the distinction between patients with familial and sporadically occurring tumors may be difficult. Approximately 50% of patients with MEN 1 die of their disease at a mean age of 51 yr, and neoplasia rather than peptic ulcer disease is the main cause of death [11].

PATHOLOGY OF MEN 1

PARATHYROID Heperparathyroidism is the most common manifestation of MEN 1, and by the age of 35 yr, its prevalence approaches 85%. Parathyroid glands of affected patients most often demonstrate evidence of chief cell hyperplasia, although examples of multiple adenomas and carcinomas have also been reported [12]. It is not surprising that hyperplasias and adenomas are coexistent in the same glands and/or different glands, suggesting that a monoclonal "adenoma" may develop

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Table 1
Multiple Endocrine Neoplasia (MEN) Syndromes

Syndrome	*Proliferative lesions of syndrome*
MEN 1	Parathyroid hyperplasia/adenoma/carcinoma, pituitary adenoma, duodenal–pancreatic endocrine cell hyperplasia/tumor (bronchial and thymic carcinoid, adrenal cortical adenoma, thyroid follicular adenoma, lipoma, angiofibroma, ependymoma)
MEN 2A	C-cell hyperplasia/medullary thyroid carcinoma, adrenal medullary hyperplasia/pheochromcytoma, parathyroid hyperplasia/adenoma
MEN 2B	C-cell hyperplasia/medullary thyroid carcinoma, adrenal medullary hyperplasia/pheochromocytoma, gastrointestinal and ocular–cutaneous ganglioneuromatosis, megacolon; Marfanoid habitus, other skeletal abnormalities

from a phase of polyclonal hyperplasia. On the other hand, molecular studies using restriction fragment length polymorphism analysis revealed that the apparently hyperplastic glands represent, in fact, monoclonal proliferations and the lesions with chromosomal deletion are larger than those without deletions, suggesting a monoclonal proliferation may develop after a phase of polyclonal hyperplasia [13,14].

It has been estimated that approx 20% of patients who present clinically with primary chief cell hyperplasia will have one of the primary MEN syndromes, most commonly MEN 1.

Parathyroid hyperplasia is characterized by an increased parenchymal cell mass. The predominant cell in this form of hyperplasia is the chief cell. Stromal fat cells are much decreased. Although primary chief cell hyperplasia may have either a diffuse or nodular pattern of growth, the latter is more common. In some cases, multifocal or nodular hyperplastic cell masses are scattered in rich fat stroma (Fig. 1). Parathyroid involvement in patients with MEN 1 is often asymmetric, and one gland may be markedly enlarged, simulating an adenoma. Amelioration of hyperparathyroidism after removal of the enlarged gland may frequently be followed by recurrent hyperparathyroidism. Subtotal parathyroidectomy is considered to be the surgical procedure of choice to treat affected individuals [15]. However, one recent report favors a conservative surgery consisting of removal of only grossly enlarged glands, as few late recurrence occur, for which there are no individual predictive criteria [16].

PANCREAS AND DUODENUM Pancreatic endocrine abnormalities are the second most common manifestations of the MEN 1 syndrome and have been reported in up to 75% of patients. The pancreatic lesions are characterized by numerous microadenomas spread through the pancreas, with occasional larger tumors (Fig. 2) [17]. The tumors consistently express multiple hormones, with one hormone usually predominant (Figs. 2 and 3) [18,19]. Tumors containing pancreatic polypeptide and glucagon are most often identified, whereas tumors expressing insulin predominantly or exclusively are less common, and tumors containing gastrin or somatostatin are extremely rare in the pancreas [18]. Pathologic findings of the pancreas of MEN 1 patients experienced in our institution are demonstrated (Figs. 4 and 5) [19].

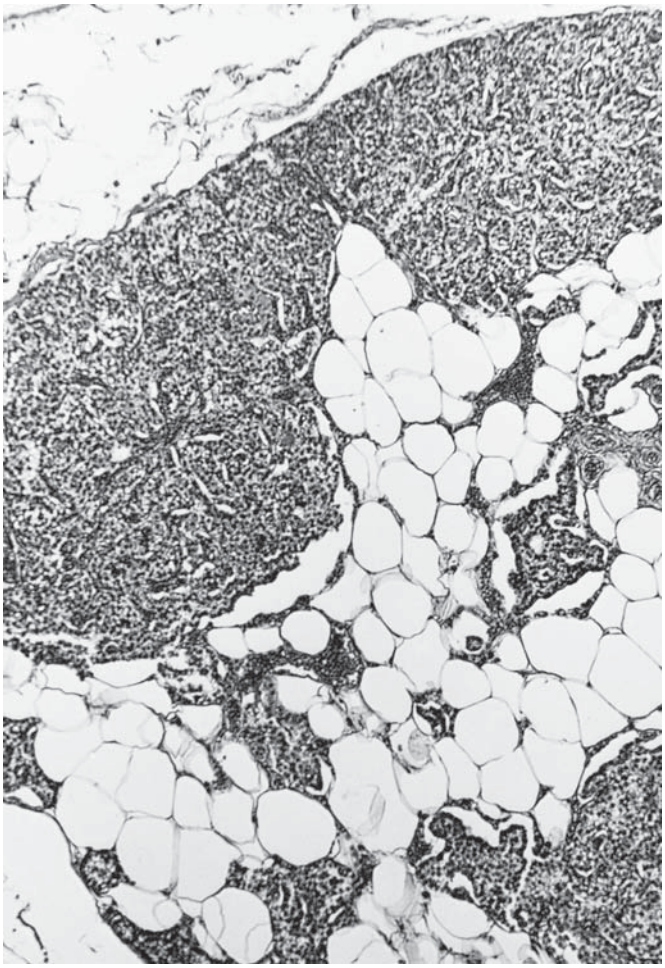

Figure 1 Multifocal and nodular hyperplastic chief cell masses in one parathyroid gland of an MEN 1 patient.

The numbers of microadenomas may vary extremely in individual cases. Tsutsumi [17] intensively studied the pancreas from a MEN 1 patient with hypoglycemia and recognized a total of 146 endocrine tumors, among which 116 glucagonomas, 14 PPomas, and 13 insulinomas, including macroadenomas maximally measuring up to 1 cm, were identified (Fig. 2). The pancreas of another MEN 1 patient with ZES and hyperparathyroidism is schematically shown in Fig. 4. In the pancreas, 3 macroadenomas, 12 microadenomas, and 2 enlarged peripancreatic lymph nodes as metastases of endocrine tumors were identified. By immunostaining and radioimmunoassay of the macroadenomas, two tumors in the pancreas were found to contain glucagon, somatostatin, and PP, and one other tumor to contain glucagon and PP. Microadenomas were all plurihormonal and mostly glucagonomas and PPomas. Gastrin and somatostatin were identified in the two metastatic lymph nodes. No gastrin was detected in the pancreas (Fig. 4) [19]. The head of the pancreas of the third MEN 1 patient without its endocrinopathy was densely studded with micro- and macroadenomas (Fig. 5), almost expressing the exocrine tissue to the periphery.

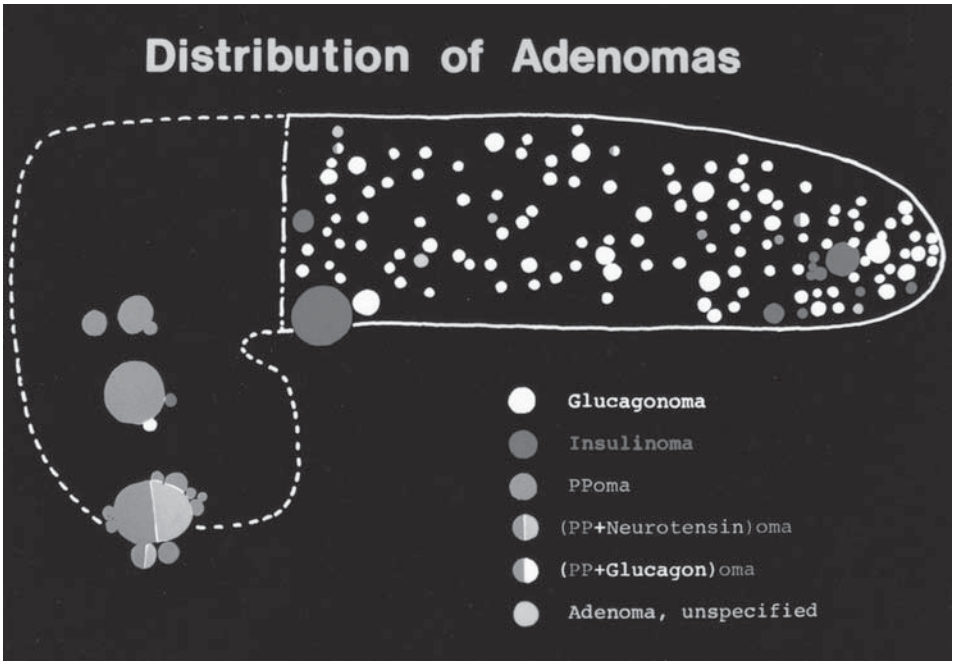

Figure 2 Distribution of 52 pancreatic endocrine tumors in a 42-yr-old female MEN 1 patient with insulinoma syndrome. (Reprinted from [17] with permission.) (Color illustration appears in insert following p. 148.)

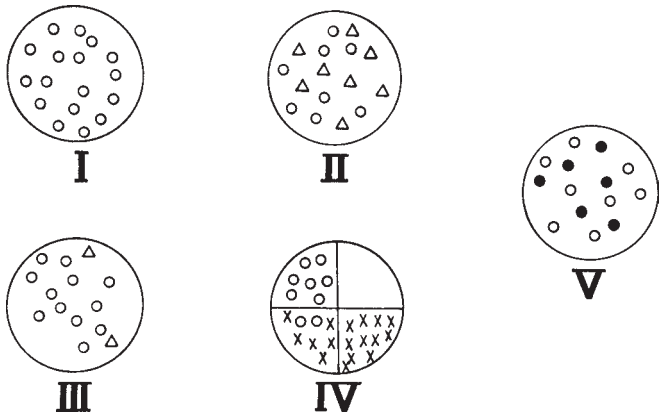

Figure 3 Features of hormone production in MEN 1 pancreatic endocrine tumors. I, Tumor in which cells produce a single pancreatic hormone; II, tumor in which separate cells produce two pancreatic hormones, respectively; III, tumor in which one cell type predominates, producing two pancreatic hormones; IV, polyclonal cell proliferations of several pancreatic hormones; V, tumor producing ectopic hormones (e.g., gastrin, ACTH, calcitonin, etc.) [19].

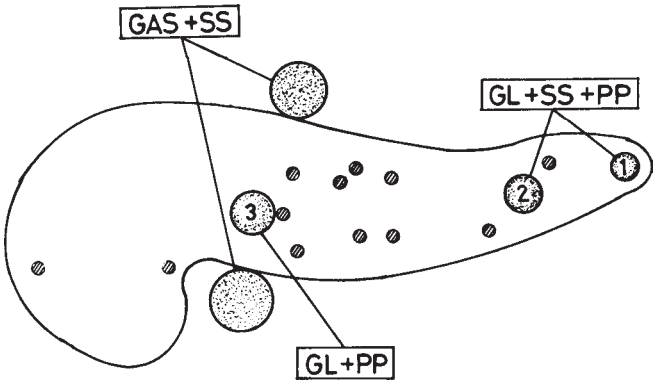

Figure 4 Distribution of 3 macroadenomas and 12 microadenomas of the pancreas and the regional node metastases in a 51-yr-old male MEN 1 patient with Zollinger–Ellison syndrome. GL, glucagons; PP, pancreatic polypeptide; SS, somatostatin; GAS, gastrin. Gastrin immunoreactivity was restricted to the two regional node metastatic tumors. All microadenomas were plurihormonal and predominantly either glucagon- or PP-producing. (Reproduced from [19] with permission.)

During the 1990s, the duodenum has increasingly been demonstrated to be the primary focus of gastrinoma with ZES in MEN 1 patients [20]. The incidence of MEN 1 associated nonduodenal and nonpancreatic neuroendocrine tumors is 5–9% [21].

In one series of 44 duodenal endocrine tumors, only three cases were associated with MEN 1 and ZES, suggesting, in general, a prevalence of non-MEN 1 duodenal endocrine tumors [22]. Sporadic gastrinomas of the duodenum are usually solitary, while MEN 1 gastrinomas are multiple at this site.

A French MEN 1 study group, using immunostaining for pancreatic hormones, examined pancreatic tumors ranging in size from 0.3 to 7 cm in 28 patients with MEN 1. Among the 100 tumors (all multiple), 7 were unclassified, 10 were plurihormonal, and 83 produced predominantly single hormones (with 50–90% of the same cell type), including 37 A (glucagon) cell tumors, 27 B (insulin) cell tumors, 11 PP cell tumors, one G (gastrin) cell tumor, and one vasoactive intestinal peptide (VIP) tumor. The ZES was present in nine patients, but there was only

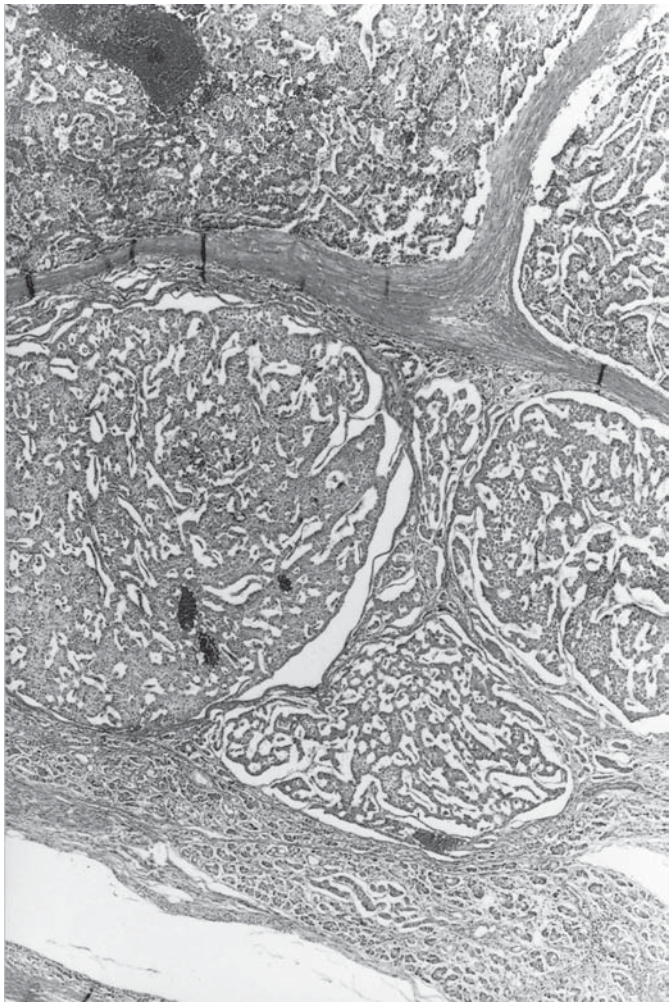

Figure 5 The pancreatic head from a male MEN 1 patient without pancreatic endocrinopathy was densely studded with micro- and macroadenomas.

one pancreatic gastrinoma. Hyperplasia of islets was not detected in adjacent pancreas and nesidioblastosis (defined as ductular proliferation of endocrine cells on the outside of the islet) was present in 30% of cases [23].

In an early study of 201 pancreatic endocrine tumors from nine MEN 1 patients, of eight patients with ZES a pancreatic gastrinoma was identified in only one patient [18]. This unexpected finding stimulated further investigations.

Malignant gastrinomas may be of two categories: one with a good prognosis because of a limited metastatic potential and tumor spread restricted to regional lymph nodes, and another with an unfavorable prognosis because of a high metastatic capacity. In cases of MacFarlane et al., most of the duodenal gastrinoma and all MEN 1 associated gastrinomas belonged to the first group. So far no histologic or other criteria of proliferative, invading, and metastatic potentials that would help to predict prognosis have been established [24].

Gastrinomas in MEN 1 are often small, multicentric, and located outside the pancreatic bed (e.g., in the submucosa of the duodenum) [20,25]. It is important to note that 50% of the gastrinomas were not detected with preoperative localization studies until before the successful development of recent methodologies of localization by endoscopic ultrasound, selective arterial secretin injection with hepatic vein sampling for gastrin, and radiolabeled octreotide scanning and surgical exploration for gastrinoma resection including detailed evaluation of the duodenum by palpation, intraoperative endoscopy with transillumination, and duodenotomy [1]. Recognition of the malignant potential of these tumors and the fact that many patients with MEN 1 have been shown to harbor multiple small, often <2 mm in diameter, lesions in the duodenal submucosa, has rekindled interest in more aggressive surgical approach aimed at "curing" these patients of their disease [26,27].

However, for the present, the role of surgical resection of gastrinomas in MEN 1 is controversial because of low biochemical cure rates, but with adequate duodenal exploration higher cure rates may be possible. The NIH group concluded from the outcome of 10 patients that not all patients with MEN 1 and ZES have duodenal gastrinomas. In the 70% of patients with duodenal tumors, even extensive duodenal exploration with removal of positive lymph nodes does not result in cures because of 86% of tumors had metastasized to lymph nodes and 43% of patients had large numbers of tumors [24].

Equally important, demonstration of a pancreatic lesion in patients with ZES does not necessarily equate with identification of the source of gastrin hypersecretion (Fig. 3). Studies in the early 1990s found numerous multicentric pancreatic tumors in patients with MEN 1 who were afflicted with ZES, while immunostaining of most of these tumors following resection revealed little or no gastrin.

Duodenal gastrinomas may give rise to periduodenal–pancreatic lymph node metastases, which can be found in 60–80% of the patients and may be much larger than the primary tumor. The development of liver metastases is rare and occurs late in the course of the disease.

In our case of "so-called lymph node gastrinoma," pancreatic and peripancreatic lesions were diagrammatically shown (Fig. 3), where no primary focus of gastrinoma was obtained even by surgery and autopsy. The most plausible explanation may be due to the nonrecognition of small tumors of either duodenum or other organs during surgery and autopsy, since the possibility of the presence of small primaries outside of the pancreas was not known at that time (in 1982) [19].

PITUITARY GLAND Pituitary adenomas, which are commonly multicentric (Fig. 6), have been reported in up to two thirds of patients with MEN 1 in combined clinical and autopsy studies [7,28–30].

Immunocytochemically, most tumors produce growth hormone (GH) and/or prolactin (PRL) and only occasionally stain for adrenocorticotrophic hormone (ACTH) [31]. Diffuse hyperplasia of one of the cell types has not yet been reported. Amenorrhea is the most frequent manifestation of pituitary lesions in women who are carriers of the *MEN1* gene. Local symptoms, for example, visual disturbance, hypopituitarism, Cushing's disease, acromegaly, or hyperprolactinemia with impotence in men are less frequently encountered [31].

Trump et al., on the other hand, reported a 30% frequency of pituitary lesions in a study of 709 individuals from 62 MEN 1 families [32]. The considerably lower rate of null-cell adeno-

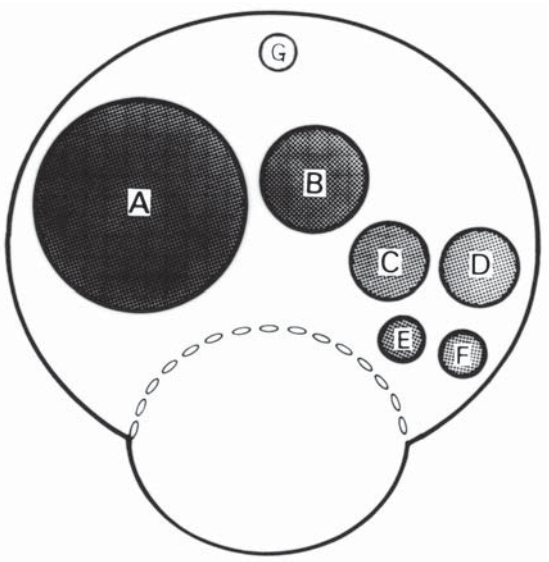

Figure 6 Whole pituitary lesions of a male MEN 1 patient were elucidated by autopsy. (**A**) PRL +++; TSH+, FSH+. (**B**) PRL+++, FSH+, LH+. (**C–F**) PRL cell proliferation in normal background. (**G**) ACTH cell aggregate.

mas among hereditary patients as compared to sporadic series (7% vs up to nearly 30%) can probably be explained by the far lower average age of MEN 1 patients [28]. Whole profiles of pituitary lesions of MEN 1 patients can be obtained only by autopsy cases in which pituitary glands could intensively be investigated. Such situations are rare [30]. Figure 6 is the entire profile of the pituitary gland of our autopsy case.

Capella et al. [30] reviewed the pathology of proliferative lesions of the pituitary gland in MEN 1. When compared with sporadic tumors occurring in the general population, adenomas with MEN 1 are more often functioning, they are more often GH- or PRL-producing, and they are more frequently plurihormonal. The study of their personal autopsy cases disclosed that pituitary PRL or mixed GH–PRL cell adenomas in MEN 1 were multiple and were associated with PRL or GH cell hyperplasia of the peritumoral parenchyma. Such hyerplasia was not observed in our case shown in Fig. 6.

A variant of MEN combining acromegaly due to a pituitary adenoma with pheochromocytoma has been reported in at least 14 cases [33].

OTHER PROLIFERATIVE LESIONS [34,35] *Neuroendocrine tumors (carcinoids)* of the lung, thymus, and stomach are considered to be an integral part of MEN 1 with either low or high penetrance in affected families. They are most frequent in the thymus, followed by the lung and stomach.

The incidence of morphological changes of the adrenal cortex in MEN 1 patients has been reported in the literature as ranging from 25% and 40%. More frequent bilateral enlargement and a predominance of hyperplasia over adenomas was found. All studies concur that the vast majority of adenocortical lesions in MEN 1 patients represent benign and endocrinologically silent processes. Adrenal carcinomas and functionally active adenomas are rare.

Thyroid diseases have been reported in 15–27% of MEN 1 patients. Included are euthyroid goiter; follicular adenomas; and papillary, follicular, and, more rarely, medullar carcinomas. Because of the high incidence of various types of thyroid changes in the normal population, it appears unlikely that the thyroid changes are causally related to the MEN 1 syndrome.

In approx 10% of MEN 1 patients, partly multifocal, mostly subcutaneous, and occasionally visceral or retroperitoneal *lipomas* have been reported.

Facial angiofibroma and *collagenoma* were recently identified in up to 88% of 32 MEN 1 patients [36]. Sixteen of the 28 patients (50%) had five or more lesions. The telangiectatic lesions are located predominantly on the nose and adjacent cheeks but can also be seen on the forehead and the upper and lower lips. Light microscopy showed that there were prominent blood vessel proliferations, concentric rings of collagen around adnexal structures and blood vessels, and stellate fibroblasts in reticular dermis. There was a trend toward increasing numbers of angiofibromas associated with increased patient age. Moreover, collagenomas (72%), café au lait macules (38%), lipomas (34%), confetti-like hypopigmented macules (6%), and multiple gingival papules (6%) were observed in these patients. It should be of note that these 32 patients with MEN 1 exhibited some of the cutaneous findings typically associated with tuberous sclerosis.

OVERVIEW OF MEN 2 [1,3,37–40]

MEN 2 syndromes are autosomal dominant inherited cancer syndromes.

Sipple's report of a single case of thyroid carcinoma and pheochromocytoma focused attention on the fact that this association was more than coincidental [4]. The patient described by Sipple was a 33-yr-old man who developed uncontrollable hypertension after surgery for an arteriovenous brain malformation. Autopsy revealed large bilateral pheochromocytomas, bilateral thyroid masses, and an enlarged parathyroid gland. A number of additional reports demonstrated that this constellation of endocrine abnormalities was inherited as an autosomal dominant trait.

The MEN 2 syndromes consist of MEN 2A, MEN 2B, and familial medullary thyroid carcinoma (FMTC or MTC-only). The principal disease characterizing these syndromes is medullary thyroid carcinoma (MTC), which occurs with complete penetrance in all affected individuals.

In MEN 2A all affected individuals develop multifocal, bilateral MTCs at a young age, usually before 30. Pheochromocytomas, which may also be multifocal and bilateral, develop in about 40% of patients. About 30% of patients develop asymmetric parathyroid hyperplasia and/or adenomas, resulting in hyperparathyroidism. Less frequently, patients may develop cutaneous lichen amyloidosis, which is characterized by brownish plaques, usually in the interscapular area. Hirschsprung's disease is also associated with MEN 2A, and is characterized by the absence of colonic autonomic ganglion cells, leading to obstruction and megacolon.

In MEN 2B, as in MEN 2A and FMTC, all affected individuals develop multifocal MTCs, but in MEN 2B the tumors tend to occur at a younger age (often <5 yr) and are more aggressive.

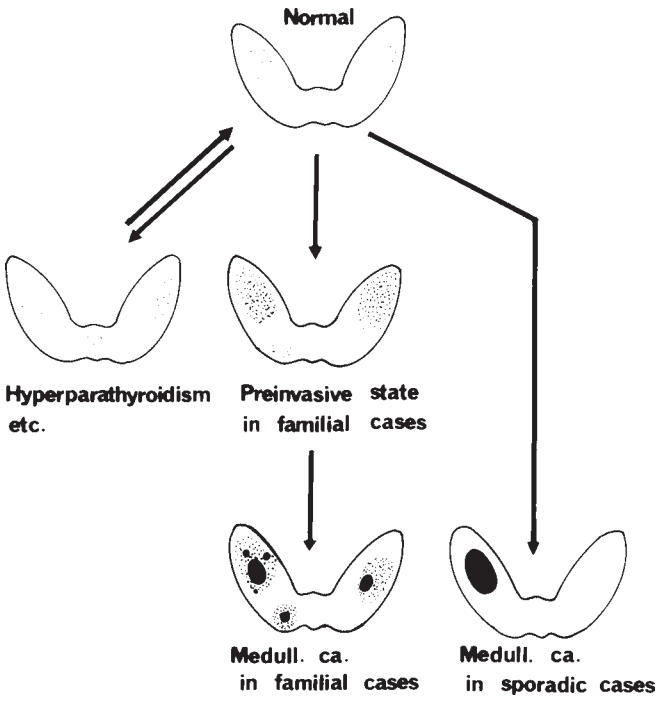

Figure 7 Tumorigenesis of C cells in familial (inherited) and sporadic cases. Preinvasive state may be called MTC *in situ*. Invasive MTCs may be small (<1 cm) and defined as microcarcinoma and may be >1 cm and defined as macrocarcinomas.

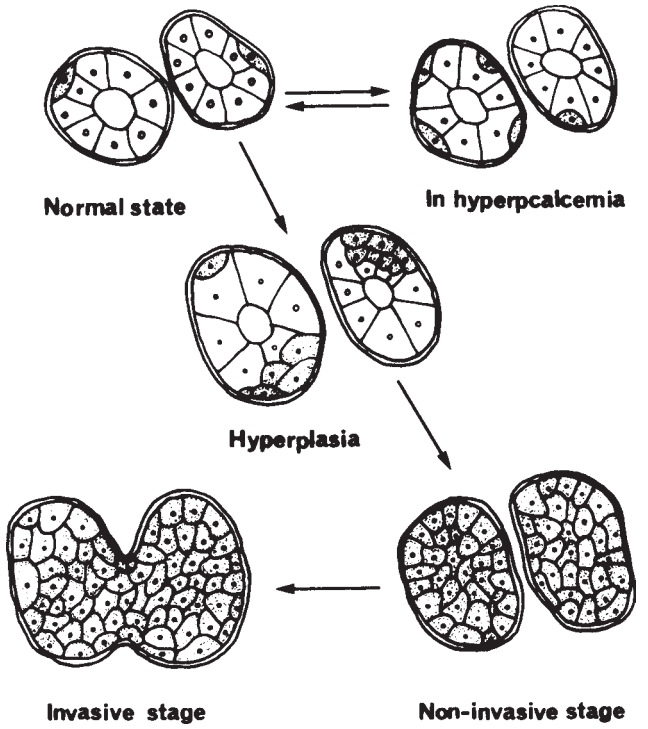

Figure 8 Processes from parafollicular C cells to invasive MTC through neoplastic hyperplasia and noninvasive stage (MCT *in situ*).

About half of MEN 2B patients develop pheochromocytoma. All individuals develop neural ganglioneuromas, particularly in the mucosa of the digestive tract, conjunctiva, lips, and tongues. MEN 2B patients have a marfanoid habitus with long axial skeletal features. Individuals with MEN 2B also develop megacolon and markedly enlarged peripheral nerves. MEN 2B patients do not develop hyperparathyroidism, as do MEN 2A patients.

FMTC is characterized by the development of MTC without any other endocrinopathies or other clinical abnormalities. These patients develop MTC at an older age, and the disease tends to be more indolent and is now considered a variant of MEN 2A, as several of these families may in fact develop pheochromocytoma if followed closely for long enough [41].

PATHOLOGY OF MEN 2

THYROID C-cell hyperplasia and MTC are usually present in MEN 2 patients. The histologic features of MTCs in patients with MEN 2A, 2B, and FMTC are identical to those of sporadic tumors. Whereas sporadic MTCs are solitary lesions unassociated with other C-cell pathology, the familial tumors arise on the background of C-cell hyperplasia. This lesion begins as an increase in the number of C cells dispersed among the follicular epithelium. The proliferating C cells replace the follicular epithelium, fill follicles, and produce small solid nodules (MCT *in situ*), and the transition to invasion occurs through the basal lamina (Figs. 7 and 8).

An estimated 25% of all MTCs are familial. Data from the German MTC Registry indicated that 16.6% are associated with MEN 2A, 5.3% with FMTC, and 2.7% are associated with MEN 2B [42]. Seventy-five percent of medullary carcinomas are nonfamilial, although this number is likely to decrease as a result of detailed genetic analyses of patients with apparent sporadic tumors.

From 1969 to 1971, total thyroidectomies were performed on the basis of abnormal basal serum levels of calcitonin. All the resected thyroids contained bilateral and multifocal MTCs, and many of them had evidence of lymph node metastases. Since calcitonin abnormalities came to be identified by calcium and/or pentagastrin stimulation, more thyroidectomies were performed; 30 resected thyroids revealed diffuse and/or nodular proliferations of C cells throughout both lobes of the thyroid (C-cell hyperplasia) in 16 patients, microscopic foci of MTC associated with C-cell hyperplasia in 13 patients, and no apparent C-cell abnormalities in only one patient [43].

As will be seen in the section on Somatic *RET* Mutation, DNA testing has eliminated the need for unnecessary biochemical screening in family members who are not at risk. This diagnostic procedure can also give predisposed family members the opportunity for surgical intervention such as prophylactic thyroidectomy before the development of MTC. Wells et al. [44] before 1994 screened 132 members of seven MEN 2A kindreds for the presence of specific *RET* mutations and identified genetic defects in 21 of 58 families at risk. Surgery was recommended to all gene carriers and accepted in 13. Of interest, although only seven of the 13 patients had positive pentagastrin stimulation tests, all had evidence of C-cell hyperplasia and several had foci of early MCT in the resected thyroid tissue. None had

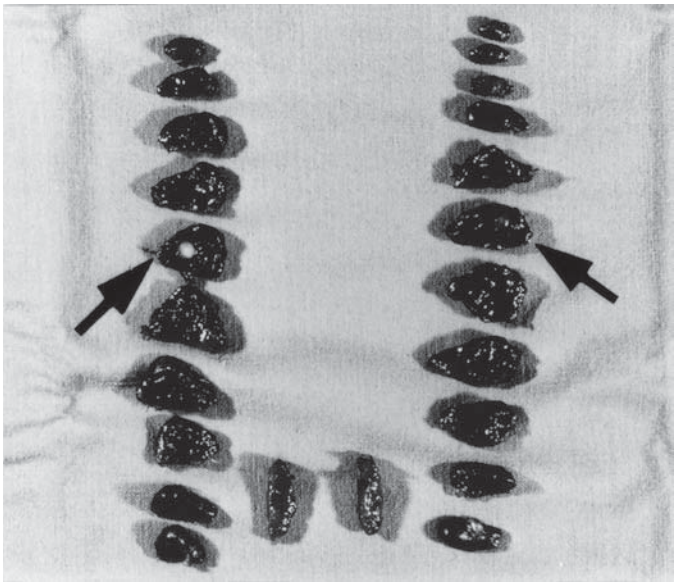

Figure 9 Horizontally sectioning of both thyroid lobes revealed bilateral gross tumors in a 26-yr-old female MEN 1 patient. In addition to these gross MTCs, examination of all the sections revealed multifocal MTCs including microcarcinomas and MTCs *in situ* and neoplastic C-cell hyperplasia.

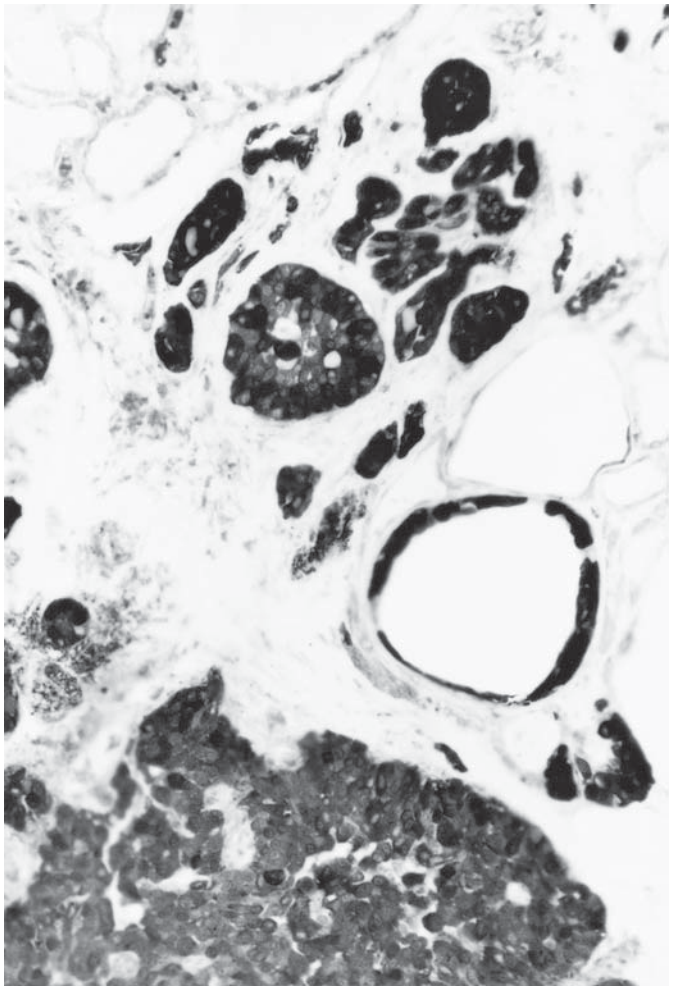

Figure 10 One invasive microcarcinoma (**bottom**) with several surrounding MTCs *in situ* and C-cell hyperplasia in one tissue section of the MEN 1 case in Fig. 9. Calcitonin immunostaining.

evidence of lymph node metastases and all have had normal pentagastrin stimulation test results postoperatively. Equally important, three members of a single kindred, previously thought to have MCT and subjected to thyroidectomy, were found by genetic analysis not to harbor the *MEN2A* gene. Review of the pathologic findings showed parafollicular cell hyperplasia but no MCT, suggesting that a false-positive stimulation test resulted in an erroneous diagnosis [44].

MTCs in MEN 2 are usually multifocal and bilateral in occurrence, and are typically preceded by bilateral, multicentric (Fig. 9), diffuse and nodular C-cell hyperplasia, which is defined as the presence of at least three low-power magnification (×100) microscopic fields containing more than 50 C cells and at least 40 cells per cm^2 [45] (Fig. 10).

There are two types of C-cell hyperplasia, one representing a physiologic or reactive process and the other a neoplastic proliferation associated with the MEN 2 syndromes or FMTC. The latter has been called medullary carcinoma *in situ* [46]. In neoplastic focal hyperplasia only part of the follicles are surrounded by calcitonin-positive C cells. The diffuse form is characterized by follicles that are lined by C cells in a ringlike fashion. In nodular hyperplasia there is obliteration of the follicles with formation of C-cell nodules surrounded by an intact basal lamina (Figs. 8 and 10). These C cells are mildly to moderately atypical, recognized by hematoxylin & eosin (H&E) stain, while it is very difficult to identify C cells of reactive hyperplasia by H&E stain. Calcitonin immunostain reveals these C cells. Distinction of intrafollicular C cells surrounded by intact basal laminae from those invading the stroma can be made by collagen IV immunostain [47]. Albores-Saavedra's group [46] has defined a tumor measuring <1 cm as medullary thyroid microcarcinoma. Although currently a rare tumor, its frequency will probably increase with the use of genetic testing for *RET* protooncogene germline mutations. Inherited medullary microcarcinomas are naturally bilateral and well or poorly demarcated but not encapsulated. Medullary microcarcinomas measuring 1–2 mm often may be overlooked. Horizontally sectioning of the thyroid lobes from the superior to the inferior poles at intervals of 2–3 mm and inclusion of all the sections for microscopic examination are recommended. With this technique, either C-cell hyperplasia or medullary microcarcinoma in thyroids of all patients with *RET* germline mutation has been identified [46] (Fig. 10).

The prognosis of inherited medullary carcinoma is much better than that of clinically apparent and larger medullary carcinomas. In 42 patients with MEN 2 who had microcarcinoma, only one patient developed lymph node metastasis and no patient died as a result of the tumor [46,48]. Kaserer et al. reported no evidence of local recurrence or distant metastases in any of 16 MEN 2 patients with microcarcinoma [49].

Patients with MEN 2A show much better survival rates than those with sporadic disease [50]. However, patients with MEN 2B syndrome are reported to have the most aggressive type of medullary thyroid carcinomas [51]. Before the introduction of the DNA test for MEN 2, the mortality in all probability was >40% [52,53], and the patients succumbed due to MCT metastases. The aggressiveness of MEN 2B may be explained by earlier MTC tumorigenesis than that of MEN 2A and sporadic MTC. The higher transforming activity of the RET protein with the MEN 2B mutation may partly explain a more aggressive phenotype of MEN 2B than MEN 2A [54].

Screening should begin at the age of 5 yr, or younger, in MEN 2A kindreds and as soon as is feasible after birth in members of MEN 2B kindreds or other kindreds historically afflicted with early appearing or particularly aggressive MCT. As the majority of patients who are going to develop MCT will have manifested the disease by the age of 35–40 yr, screening should continue at least until that age [1].

MEDULLA OF THE ADRENAL GLAND As in MCTs in patients with MEN 2 syndromes, pathologic change of the adrenal medulla is a stepwise progression from hyperplasia to neoplasia with frequent occurrence of multicentric and bilateral pheochromocytomas [55].

The most notable cases of familial pheochromocytomas are found in MEN 2A and MEN 2B patients, but they can occur with von Recklinghausen's disease (about 5%) [56] and von Hippel–Lindau disease (10–20%) [57]. A case of bilateral composite pheochromocytoma–ganglioneuroma was reported in a patient with type 1 neurofibromatosis [58].

Pheochromocytomas develop in 30–50% of patients with MEN 2 [59]; the tumors are bilateral or multifocal in about 60% [60]. The tumors tend to produce more epinephrine, in contrast to sporadic pheochromocytoma in which norepinephrine is the predominant catecholamine secreted [59,61].

Some studies have suggested that these tumors represent an extreme degree of adrenal medullary hyperplasia [62]. Malignant pheochromocytomas may be relatively rare in MEN 2 [59,60].

Familial adrenal medullary hyperplasia (FAMH) has been well established in the MEN 2A and 2B, is regarded as the precursor of pheochromocytomas, and may coexist with pheochromocytomas. The diagnosis of AMH is sometimes difficult and the distinction between AMH and pheochromocytoma may occasionally be impossible.

Nodular or diffuse hyperplasia is a characteristic feature of MEN 2A and 2B, and the pheochromocytomas are frequently multicentric and bilateral. AMH can be symmetric or asymmetric in both adrenals and occasionally mild, the glands appearing almost normal.

In AMH nodules, there may be compression of adjacent cortex by nodular expansion of the medulla. Frank invasion is lacking and there is no histologic evidence of encapsulation.

One of the early indications of adrenal medullary hyperfunction in MEN 2 is an elevated ratio of epinephrine to norepinephrine in urine [61].

PARATHYROID GLAND Hyperparathyroidism is the least common manifestation of MEN 2A and occurs rarely, if at all, in patients with MEN 2B. Parathyroid abnormalities, including hyperplasia and adenoma, occur in fewer than 20% of MEN 2A patients. In studies by Gagel et al. [61], parathyroid hormone levels of 22 MEN 2A patients did not differ significantly from those in unaffected individuals, and none of these patients studied after thyroidectomy had evidence of hyperparathyroidism.

GASTROINTESTINAL TRACT Hirschsprung's disease has been reported in a small number of kindreds with MEN 2A [63]. Identification of mutations in the *RET* gene locus (*see* the section on Germline Mutations of the *RET* Gene) that are linked to the MEN 2 syndromes raises the question of the role of the *RET* gene product in the development of the observed clinical phenotype. Other studies, establishing linkage between the *RET* gene locus and a form of genetic Hirschsprung's disease have provided insight into the molecular derangements that underlie these syndromes. Examination of the *RET* coding sequence in individuals from Hirschsprung kindreds revealed mutations, some of which reflected substantial deletions in the *RET* coding sequence, suggesting that Hirschsprung's defect reflected a loss-of-function mutation of the *RET* gene, while MEN 2 syndrome reflected a gain-of-function of the same gene. Hirschsprung's disease is characterized by the failure of myenteric ganglia to develop in impaired gut motility and, in severe cases, megacolon [1,64], while MEN 2B is associated with marfanoid habitus, neuromas of the lip and conjunctivae, medullated corneal nerve fibers, and disordered autonomic ganglia in the wall of the gut and other viscera [63]. A study of the dual effect on the RET receptor of MEN2 gene mutations affecting specific extracytoplasmic cysteines of the *RET* protein suggests that mutations of cysteines 609, 618, and 620 of the *RET* gene exert both activating and inactivating effects [65].

MOLECULAR ADVANCES IN MEN 1 AND MEN 2

STRUCTURE AND FUNCTION OF THE *MEN1* GENE

The human *MEN1* gene consists of 10 exons distributed over seven kilobases (kb) in the chromosome region 11q13, and encodes mRNA of approx 2.8 kb coding for a 610-amino-acid protein, referred to as menin (Fig. 1) [66,67]. The primary structure of menin is not similar to previously identified proteins, thus providing few clues to its normal function. Menin mRNA is expressed in all tissues and cell lines examined, but the expression levels are not uniform among different tissues [68–73]. The expression is high throughout the early embryo and in several adult tissues including central nervous system, thymus, testis, and placenta. Menin homologs have been identified in other species including the mouse [68,70,71], rat [70,71], zebrafish [74,75], fruit fly [76,77], and snail [78], but not in the yeast *Saccharomyces cerevisiae* [77] and nematode *Caenorhabditis elegans* [77].

The *MEN1* gene is a tumor suppressor gene and at least one copy of the normal gene is required to prevent tumor development. Indeed, various heterozygous germline mutations of the *MEN1* gene, which cause loss of the gene function, have been identified in the majority of MEN 1 patients [66,67,79–83]. In addition to germline mutations, somatic loss of the normal *MEN1* allele has almost constantly occurred in the tumors associated with MEN 1, in agreement with the Knudson's two-hit model for tumor development [84]. Somatic inactivation of both *MEN1* alleles is also observed in a fraction of sporadic *MEN1*-

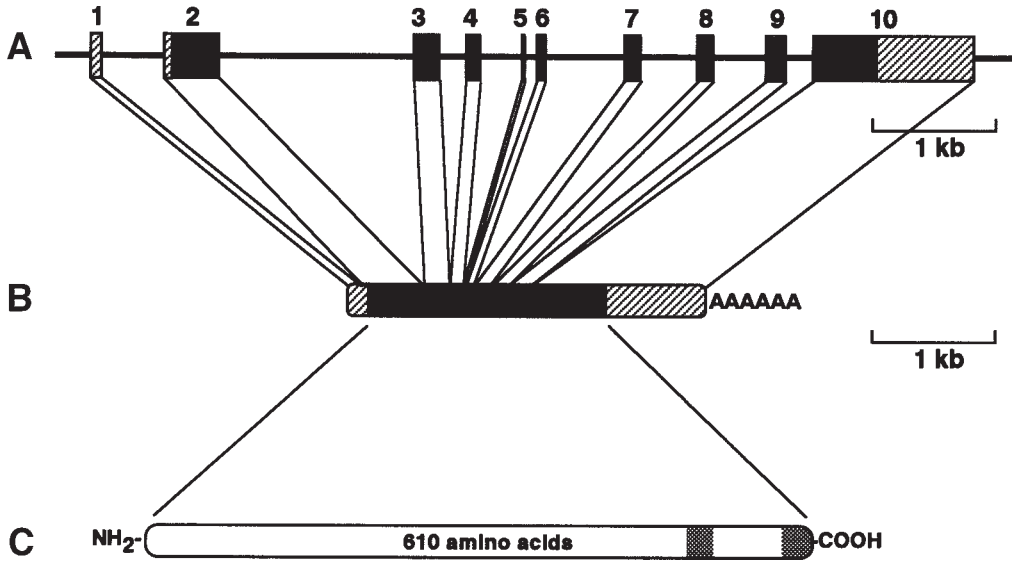

Figure 11 Structural organization of the human *MEN1* gene and its products. (**A**) *MEN1* gene. *Closed* and *hatched boxes* indicate protein-coding and noncoding regions of exons, respectively. (**B**) Menin mRNA. *Closed* and *hatched boxes* indicate translated and untranslated regions of the mRNA, respectively. (**C**) Menin. *Shaded boxes* indicate nuclear localization signals [86].

related tumors such as sporadic parathyroid tumors and gastrinomas, indicating the involvement of this gene in the development of a subset of sporadic tumors. Heterozygous germline disruption of the *MEN1* gene in mice produced a phenotype remarkably similar to human MEN 1 [85].

Menin has amino acid sequences necessary for nuclear localization at its carboxy-(C)-terminal region (Fig. 11), and is localized mainly to the nucleus [86,87]. Menin has been shown to interact with transcriptional regulators JunD [88–90], NF-κB [91], and Smad3 [92], with the putative tumor metastasis suppressor nm23 [93], and with the homeobox-containing protein Pem [94]. Transcriptional activation by JunD and NF-κB is repressed by menin, while Smad3-induced transcription appears to be enhanced by menin. Antisense menin RNA has been shown to antagonize Smad3-induced and transforming growth factor-β (TGF-β)-induced transcriptional activation and TGF-β-mediated inhibition of cell proliferation. Menin has also been implicated in synapse formation between neurons [78]. These findings suggest that menin is involved in cell signaling for the regulation of cell proliferation and differentiation.

GERMLINE MUTATIONS OF THE *MEN1* GENE

Detection Rate of Mutation Heterozygous germline mutations of the *MEN1* gene have been identified in about 80% of familial MEN 1 and about 50% of sporadic MEN 1 patients [66,67,79–83]. These limited detection rates result from imperfect analytical methods as well as heterogeneity of diseases diagnosed as MEN 1. Mutation screening by rapid methods such as single-strand conformation polymorphism analysis [82], heteroduplex analysis [83], and denaturing gradient gel electrophoresis [95] may miss some mutations that could be identified by direct sequencing. Analysis of only limited regions of the gene, usually protein coding exons and its short flanking sequences, cannot detect mutations in other regions that may affect the gene transcription or mRNA splicing. Even if a nucleotide change is identified in these regions, it is difficult to distinguish a causative mutation from a benign polymorphism because the biological significance of the nucleotide alteration is not obvious [96]. Also, large deletions would escape detection by the standard analysis methods [97,98].

Heterogeneity of the diseases diagnosed as MEN 1 is another cause of the imperfect detection rate. A kindred with atypical MEN 1 demonstrating frequent expression of pituitary tumors and a low penetrance of primary hyperparathyroidism in contrast to classical MEN 1 has been shown not to link to the *MEN1* locus, indicating that this variant of MEN 1 is a disease entity distinct from classical MEN 1 [99]. The lower detection rates in sporadic MEN 1 than those in familial MEN 1 probably reflect a higher incidence of phenocopy in the cases diagnosed as sporadic MEN 1 [100].

Germline mutations of the *MEN1* gene have been identified in a few patients with clinical presentations suggestive of MEN 1 but not diagnosed as MEN 1 according to the criteria. A small number of familial isolated hyperparathyroidism (FIHP) families [81,98,101–108] and an even smaller number of patients with apparently sporadic parathyroid tumors [98,109,110] have been reported to have a germline mutation. One patient with sporadic adrenocortical tumor [111], one with sporadic insulinoma [112], and another with sporadic pulmonary carcinoid [113] have also been shown to carry a germline mutation. Some sporadic tumor cases with a germline *MEN1* mutation turned out to be classical MEN 1 afterward [109,111,113]. Familial isolated pituitary tumor has not been associated with *MEN1* gene mutations [98,105,114–118], suggesting that this syndrome is a disease entity distinct from MEN 1.

Distribution and Type of Mutation Germline mutations of the *MEN1* gene so far identified in MEN 1, FIHP, and apparently sporadic parathyroid tumor are summarized in Fig. 12. Mutations are scattered throughout the entire protein coding exons and their splicing junctions. No clear mutation hot spot

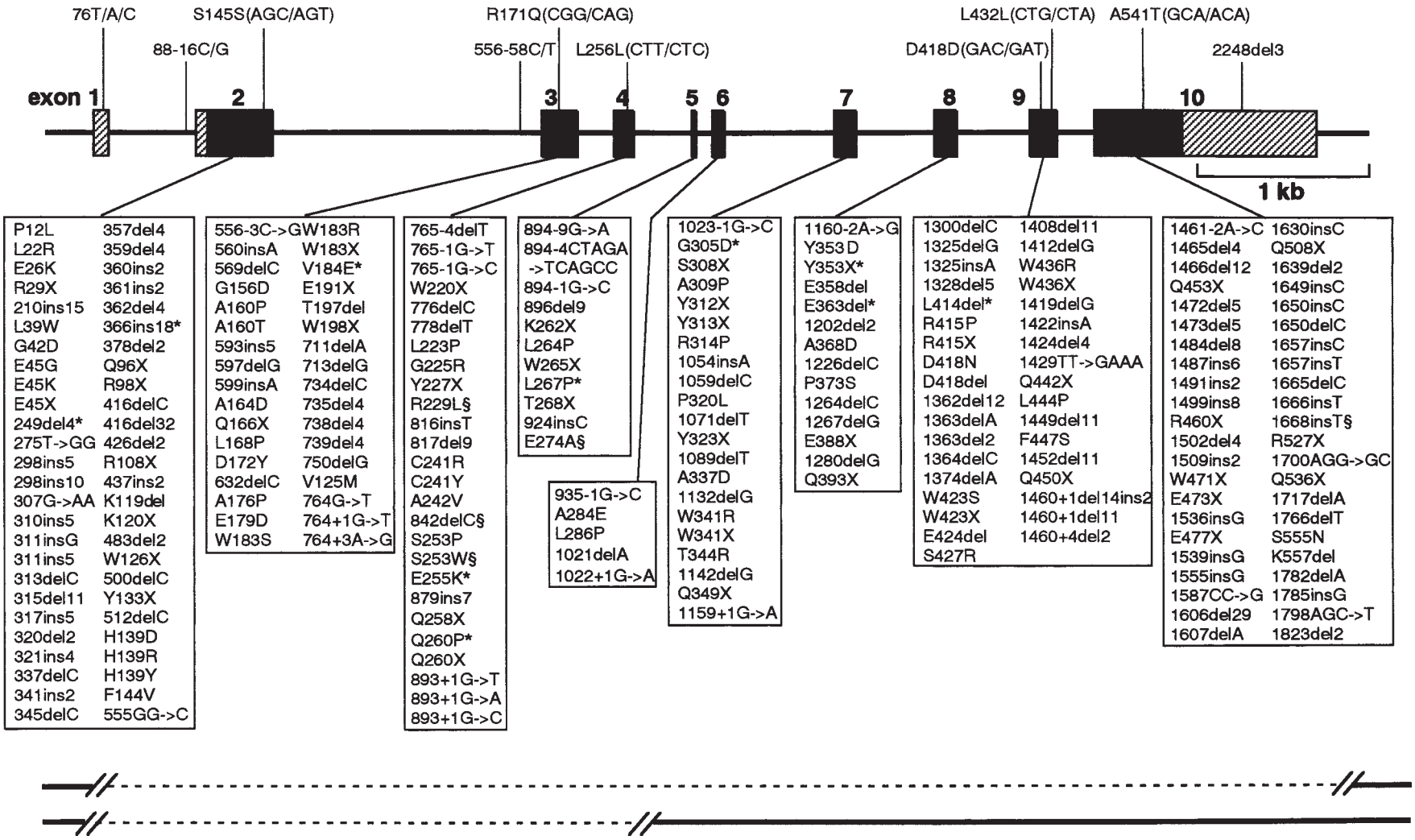

Figure 12 Distribution of germline *MEN1* gene mutations identified in patients with MEN 1 and related disorders. *Closed* and *hatched boxes* indicate protein-coding and noncoding regions of *MEN1* gene exons, respectively. Mutations identified in each exon and its adjacent intron sequences are shown together in a *box* below. Benign polymorphisms are shown *above*. The symbols * and § indicate mutations identified in patients of FIHP and apparently sporadic parathyroid tumor, respectively. Mutation abbreviations follow standard nomenclature [127]. *Broken lines below* depict large deletions [97,98].

Table 2
Germline *MEN1* Gene Mutations
Identified in Patients with FIHP and Sporadic Parathyroid Tumor

Phenotype	*Mutation*[a]	*Effect on Menin*	*References*
FIHP	*249del4*	Frameshift, at codon 47	[101
	366ins18	Six insertions of amino acids at codon 86	[98]
	V184E	Amino acid substitution	[102]
	E255K	Amino acid substitution	[103]
	Q260P	Amino acid substitution	[104]
	L267P	Amino acid substitution	[105]
	G305D	Amino acid substitution	[106]
	Y353X[b]	Protein truncation at codon 353	[81]
	E363del	One amino acid deletion at codon 363	[107]
	L414del	One amino acid deletion at codon 414	[108]
Sporadic parathyroid tumor	*R229L*	Amino acid substitution	[98]
	842delC[c]	Frameshift at codon 245	[110]
	S253W	Amino acid substitution	[109]
	E274A	Amino acid substitution	[109]
	1668insT[b]	Frameshift at codon 520	[109]

[a]Mutation abbreviations follow standard nomenclature [127].
[b]The family was diagnosed as having MEN 1 afterwards (Shimizu et al., personal communication and [109]).
[c]The patient had multiple adenomas and carcinomas.

has been identified, but some mutations have been repeatedly encountered in apparently unrelated families. Some of them appear to be founder mutations originating from the common ancestors as evidenced by common chromosome haplotypes [119,120]. However, patients of different ethnic groups often share identical mutations at these "warm spots." Furthermore, several recurrent mutations link to different haplotypes, indicating that not all of them are founder mutations. These mutations are often insertions or deletions at a repetitive nucleotide sequence, suggesting that they might occur more frequently than other mutations possibly by slippage-mediated mutation during DNA replication [119].

Various mutations including single nucleotide alterations, insertions, and deletions have been identified. Approximately 50% and 15–20% of the *MEN1* mutations are frameshift and nonsense mutations, respectively, both of which cause truncation of menin. Splicing mutations usually recognized as a nucleotide change of the donor (GT) or the acceptor (AG) consensus sequences account for 5–10% of the mutations. Large deletions encompassing the entire or partial gene regions have rarely been demonstrated [97,98].

Except for splicing mutations occurring outside the GT/AG consensus sequences, all the mutations mentioned above are easy to interpret as a pathogenic mutation when they are detected. On the other hand, missense mutations and in-frame insertions and deletions, which collectively account for 20–30% of the *MEN1* mutations, are often difficult to interpret because they might represent benign polymorphism. Cosegregation of the mutation with disease should be carefully examined before its etiological significance is concluded.

Genotype–Phenotype Correlation No clear phenotype–genotype correlation has been established. A subset of MEN 1 with unusually high frequency of prolactinoma and a low frequency of enteropancreatic endocrine tumor has been described as MEN 1_{Burin} or the prolactinoma variant of MEN 1 [121]. Three distinct truncating mutations, *Y312X, R460X*, and *1021delA*, have been identified in MEN 1_{Burin} [80,122,123]. These mutations do not appear to be very different from other nonsense mutations causative of classical MEN 1. Recently, a Japanese prolactinoma-prone MEN 1 family has been shown to have germline mutation *357del4* [124], which was previously reported to be associated with classical MEN 1 [66,67,83,116]. Thus, it is unlikely that particular *MEN1* mutations are specific for MEN 1_{Burin}, and the molecular basis of high penetrance of prolactinoma in certain MEN 1 families is still an enigma.

A small number of families affected with FIHP have been shown to carry germline *MEN1* mutations [81,98,101–108]. Because hyperparathyroidism is the most prevalent and early expression of MEN 1, families showing the phenotype of FIHP may display the manifestations of MEN 1 subsequently, and the mutations identified in these families may not be specific to the FIHP phenotype. However, the mutations in FIHP families are mostly missense mutations or in-frame deletions or additions (Table 2), while the majority of mutations in the typical MEN 1 are frameshift and nonsense mutations. Some mutation carriers in these FIHP pedigrees were devoid of any signs of primary hyperparathyroidism even at the relatively advanced age. Moreover, most of the germline *MEN1* mutations identified in patients with apparently sporadic parathyroid tumor are also missense mutations (Table 2). These findings suggest that menin mutants identified in FIHP and apparently sporadic parathyroid tumor may retain some tumor suppressor activity and may cause these MEN 1 related disorders as milder expressions of MEN 1.

DNA TEST FOR MEN 1 The DNA test for MEN 1 is of benefit to suspected mutation carriers including patients of endocrine tumors suggestive of MEN 1 and offspring of MEN 1 patients. The DNA test in suspected individuals who have symp-

toms suggestive of MEN 1 usually requires mutation screening over the entire *MEN1* gene region, because they are usually the first to be examined in their families. Absence of any mutation does not completely exclude the diagnosis of MEN 1. The presence of a pathogenic mutation indicates that they must be treated as MEN 1 patients who should be examined for MEN 1 related tumors and informed of the hereditary nature of the disease. The procedures for tumor resection and follow-up of mutation-positive patients are different from those for sporadic tumors [125]. Some experts recommend the DNA test of patients with apparently sporadic parathyroid tumor before parathyroid surgery [109].

The benefit of the DNA test seems to be obvious when it is used to determine whether or not an apparently healthy offspring of MEN 1 patients has a predisposition to the disease. The DNA test could release nonmutation carriers from unnecessary medical examinations and fear of the disease. However, the DNA test for *MEN1* mutation is less urgent compared with that for *RET*, because positive results of the *MEN1* test do not lead to effective intervention of tumor development as opposed to the *RET* mutation test [126].

STRUCTURE AND FUNCTION OF THE *RET* GENE

The *RET* protooncogene was originally isolated by the transfection of NIH 3T3 cells with DNA from a human T-cell lymphoma [128]. Subsequent studies revealed that the gene has 21 exons and more than 60 kb of genomic DNA [129,130]. The gene encodes a receptor tyrosine kinase, which uniquely has a cadherinlike domain in the extracellular region (Fig. 12) [131, 132]. The *RET* gene generates up to 10 different protein isoforms, which result from alternative splicing. The *RET* gene localizes at chromosome 10q11.2, in which the susceptibility gene for MEN 2 has been traced [133,134].

Physiological Function of *RET*

The *RET* gene is expressed in various tissues including peripheral nervous system, central nervous system, and urinary system during development [135]. Knockout mouse studies revealed that the homozygous mutant exhibited an absence of the myenteric neurons in the small and large intestines, the esophagus, and stomach, and also absent or rudimentary kidneys and showed severe dysplasia or absent ureters [136]. These suggest that *RET* is essential for the development of the enteric nervous system and urinary system. However, the function of *RET* during normal human development remains to be determined. The *RET* gene was largely expressed in neural crest-derived cells including calcitonin-producing C cells in the thyroid and adrenal medulla and Schwann cells after birth but its expression level is low.

Ligand for *RET* Protein

The ligands for the RET are glial cell line-derived neurotrophic factor (GDNF) [137,138] and neurturin (NTN) [139]. These ligands are members of glial cell line-derived neurotrophic factor family ligand (GFL) including other ligands of artemin [140] and persephin [141]. These ligands form the multiple complex with glycosyl phosphatidylinositol-anchored protein, GDNF family receptor-α (GFR-α), which is fixed to the cellular membrane. These RET complexes formed dimers and autophosphorylated, resulting in signaling pathways to the nucleus. So far, GFR-α-1, -2, -3, and -4 have been identified as GFR-α, although these ligands exhibit a higher affinity for GDNF, NTN, artemin, and persephin, respectively [142].

Germline Mutation of the *RET* Gene

In more than 95% of MEN 2A or MEN 2B families, germline missense mutations have been found [143,144]. In FMTC families, a germline missense mutation was identified in approx 90% of the family. Gain-of-function mutation in one of the two alleles exhibits tumors arising from the C cell of the thyroid, medulla of the adrenal gland, or parathyroid gland. The loci of the mutations were clustered at hot spots in the *RET* gene, and a genotype–phenotype correlation was observed. The distribution pattern of the hot spot varies among each subtype of MEN 2A, MEN 2B, and FMTC. In the majority of MEN 2A and FMTC patients, these mutations are clustered in five cysteine codons in the cysteine-rich extracellular domains of the *RET* C609, C611, C618, and C620 of exon 10, and C634 of exon 11 (Fig. 13 and Table 3). Mutations of these codons have been detected in 95% of MEN 2A cases and also found in about 85% of FMTC families. Moreover, mutations at cysteine codon 634 represent 85% of all *RET* mutations and appear predictive of the presence of the pheochromocytoma.

In rare cases, missense mutations at codons 768 and 804 have been found in low penetrance cases of the diseases [145, 146]. In other rare cases, germline missense mutations at codons 630, 631, 790, 791, and 891, an insertion of 9 basepairs between codons 633 and 634, and an insertion of 12 basepairs between codons 634 and 635 have been found in MEN 2A or FMTC [147–149]. On the other hand, in the majority of MEN 2B cases, a germline missense mutation occurs at codon 918, where methionine to threonine substitution, ATG $\rightarrow$ ACG transversion, has been found. In another rare case, germline missense mutation at codon 883 was reported [150].

SOMATIC *RET* MUTATION

MTC or pheochromocytoma, which is the component of tumors occurring in MEN 2, has been observed sporadically, where germline *RET* mutation could not be detected. One-third or two-thirds of the sporadic MTC cases harbor a *RET* mutation in the tumor as a somatic mutation but not as a germline mutation. In most of the MTCs with a somatic mutation, the *RET* mutation has been located at codon 918 [145,149,151–153]. In 10–20% of sporadic pheochromocytomas, the *RET* mutation has been found as a somatic mutation [154–156]. In most of them, the codon 918 mutation was found. These suggest that the *RET* gain of function of mutations are closely associated with not only hereditary MEN 2 but also nonhereditary sporadic cases of MTC or pheocromocytoma.

DNA TEST FOR MEN 2

Mutational analysis of the *RET* gene not only can confirm the diagnosis of MEN 2 but can also identify asymptomatic family members at risk for this syndrome. Before DNA testing was available, both affected and unaffected individuals at 50% risk of MEN 2 were required to undergo annual biochemical screening for MTC, pheochromocytoma, and hyperparathyroidism (HPT) from early childhood. These screening tests include pentagastrin or combined pentagastrin–calcium provocative test, which often generated a false-positive or a false-negative result in addition to patients' physical discomfort. DNA testing, which is accurate and sensitive and does not have physically adverse effects on patients, has resolved these problems, and has eliminated the need for unnecessary biochemical screening in family members who are

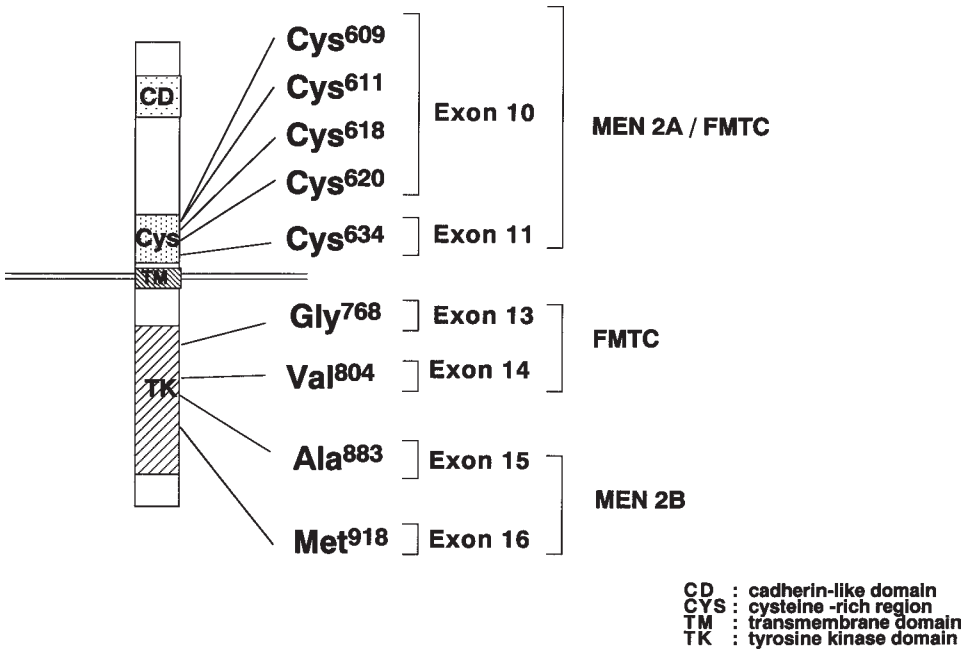

Figure 13 Structure of RET protein and representation of mutated codons in MEN 2 subtypes.

Table 3
Mutated Codons and Their Frequencies in MEN 2 Subtypes

Exon	*Codon*	*MEN 2A*	*MEN 2B*	*FMTC*	*Other*	*Total*
	609	1 (0.5)[a]		2 (6)	2 (1)	5 (1)
10	611	5 (2.5)		1 (3)	7 (4.4)	13 (3)
	618	10 (5)		10 (29)	18 (11)	38 (8)
	620	14 (7)		5 (15)	13 (8)	32 (7)
11	634	169 (83)		9 (26)	93 (58)	271 (57)
12	768			3 (9)	1 (0.6)	4 (0.8)
13	804				2 (1)	2 (0.4)
14	918		75 (95)			75 (16)
No mutation		4 (2)	4 (5)	4 (12)	25 (16)	37 (7.8)
Total		203 (100)	79 (100)	34 (100)	161 (100)	477 (100)

From ref. [144].
[a]The numbers in parentheses represent percentage of the given mutations in the MEN 2 subtypes.

not at risk. This diagnostic procedure can also allow predisposed family members the opportunity for surgical intervention such as prophylactic thyroidectomy before the development of MTC. Although MTC occurs in more than 95% of patients with MEN 2 and is potentially lethal, it can be prevented by prophylactic thyroidectomy. The thyroidectomy is safe when an experienced surgeon performs the surgery and thyroid replacement hormone is available.

The time at which the prophylactic surgery should be performed as well as the family members who should undergo the surgery are still controversial, however. Skinner et al. recommended that prophylactic thyroidectomy should be performed at the age of 5 in individuals at risk for MEN 2A and at an earlier age in those at risk for MEN 2B because of the higher virulence and earlier onset of MTC [157]. Lips et al. suggested that the surgery for individuals at risk for MEN 2A can be postponed until the positive results of the provocative test or the age of 12–13 yr [158]. However, it is recommended for the present that the timing of thyroidectomy should be performed earlier [159].

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20 Polyglandular Autoimmune Syndromes

RICARDO V. LLOYD, MD

INTRODUCTION

Polyglandular autoimmune syndrome (PGA) includes a complex mixture of endocrine and nonendocrine disorders. PGA is generally divided into type I and type II disorders (Table 1) [1,2].

PGA TYPE I

PGA I is also termed autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED). Patients with PGA I usually have mucocutaneous candidiasis, autoimmune hypoparathyroidism, and Addison's disease. The disease is usually present in early childhood with the appearance of chronic mucocutaneous candidiasis first with subsequent development of hypoparathyroidism and Addison's disease. There is an equal sex incidence [3,4]. Because the disorder may appear many years apart, long-term follow-up is usually required in the management of these patients. In one of the larger series of patients studied, 100% of individuals developed chronic candidiasis, while 79% had hypoparathyroidism and 72% Addison's disease [3]. All three of these disorders were present in 51% of patients [3]. Because of the universal development of chronic candidiasis, a T-cell function defect has been proposed in the pathogenesis of PGA I [1].

Patients with PGA I usually have antibodies against the parathyroid and adrenal glands [5]. Antibodies against 17-hydroxylase (CYP17) and side chain cleavage enzyme have been reported in Addison's disease associated with PGA I [6–8]. This is in contrast to PGA II, in which autoantibodies against 17-hydroxylene (CYP17) and side chain cleavage enzyme (CYP11A1) are commonly found [6,7].

Most patients have antibodies to glutamic acid decarboxylase (GAD65). This antibody may be detected up to 8 yr before development of diabetes mellitus.

GENETICS PGA I is inherited as an autosomal recessive disorder. There is a 25% recurrence risk for siblings of affected patients [9]. The disease is highly prevalent in Finland and parts of Italy [10]. The molecular genetics of PGI I has been elucidated [11–14].

The autoimmune regulator (*AIRE*) gene is located on chromosome 21q 22.3. More than 45 different mutations in the *AIRE* gene have been identified and are distributed throughout the entire coding region. Several of the *AIRE* mutations predict the transcription and translation of a truncated protein, which may be nonfunctional [12]. Analyses of APECED in all of the autoimmune conditions typically associated with APECED have failed to show a conclusive role of a single genetic locus capable of providing insight into the etiology of PGA I [12]. A recent study examined mutations of *AIRE1* coding to determine if the heterozygous state predisposed to more common isolated autoimmune endocrinopathies such as Addison's disease, type 1 diabetes mellitus, Graves' disease, and Hashimoto's thyroiditis [13]. Analysis for mutation *R257x* in exon 6 and a 13-basepair (bp) depletion in exon 8 showed that although some mutations in exon 6 or 13-bp deletion were found, these *AIRE1* mutations were so rare in the general population that they could not contribute to susceptibility for the more common isolated autoimmune disorders [13].

PGA TYPE II PGA II is more common than PGA I. It is usually associated with the HLA region on the short arm of chromosome 6 (6p21.3) and develops in older patients compared to PGA I. There is a female preponderance with a peek incidence between ages 20 and 60. It has also been designated as Schmidt's syndrome, polyglandular failure syndrome, and organ-specific autoimmune disease. PGA II is often defined by the development by two or more of the following conditions: Addison's disease, Graves' disease, autoimmune thyroiditis, type 1 diabetes mellitus, myasthenia gravis, celiac disease, or primary hypogonadism. In one study of 224 patients with Addison's disease and PGA II, type 1 diabetes mellitus was present in 52% and autoimmune thyroid disease in 69%. Vitiligo and gonadal failure were present in only 5% and 4%, respectively [4]. The development of hypoparathyroidism is uncommon in PGA II, unlike in PGA I, although it may be present in a small number of older patients [15]. Patients do not develop mucocutaneous candidiasis.

GENETICS There is no clear pattern of inheritance for PGA II, although familial clustering has been noted. Multiple genetic loci, especially HLA, probably determines susceptibility [1]. The HLA haplotypes involved include *HLA-A1, HLA-B8, HLA-DR3*, and *HLA-DR4*. Interestingly, some HLA alleles are associated with protein from disease such as the DQ alleles *DQA1*, *DQB2* which protect against diabetes mellitus, but ironically increase susceptibility to multiple sclerosis [16].

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Table 1
PGAs—Endocrine Tissues that Are Frequently Involved

Disorder	*Prevalence (%)*
Type I[a]	
Hypoparathyroidism	89
Chronic mucocutaneous candidiasis	75
Adrenal insufficiency	60
Gonadal failure	45
Hypothyroidism	12
Insulin-dependent diabetes mellitus	1
Hypopituitarism	<1
Diabetes insipidus	<1
Type II[b]	
Adrenal insufficiency	100
Autoimmune thyroid diseases	70
Insulin-dependent diabetes mellitus	50
Gonadal failure	5–50
Diabetes insipidus	<1

Compiled from refs. [1] and [2].

[a]Other conditions associated with PGA I include chronic active hepatitis, malabsorption, oral squamous cell carcinoma, alopecia, vitiligo, pernicious anemia, pure red cell hypoplasia, myopathy, keratopathy, and asplenism.

[b]Other conditions associated with PGA II include: celiac disease, alopecia, vitiligo, dermatitis herpetiform, pernicious anemia, idiopathic thrombocytopenia, purpura, myasthenia gravis, Parkinson's disease, IgA deficiency, and Goodpasture syndrome.

Some components of PGA II are not associated with *HLA-DR3* [17]. These include pernicious anemia, vitiligo, and multinodular thyroiditis.

The gene responsible for PGA II has not been characterized to date but will probably be linked to the HLA loci.

TREATMENT The treatment of patients with PGA I is directed at the specific abnormalities. Mucocutaneous candidiasis is treated with antifungal agents; adrenal insufficiency and hypoparathyroidism are treated with replacement medications; and hypocalcemia is treated with restoration of calcium levels including use of magnesium for hypomagnesemia [1].

PGA II therapy includes treatment of the individual disorders such as diabetes mellitus, Addison's disease, Graves' disease, and hypogonadism [1].

MISCELLANEOUS PGSs

THYMIC DISORDERS Disorders associated with the thymus include myasthenia gravis, red blood cell aplasia, hypoglobulinemia, autoimmune thyroid disease, and adrenal insufficiency. DiGeorge syndrome includes congenital aplasia or hyperplasia of the thymus and parathyroid glands, which are derived from the third and fourth pharyngeal pouches. DiGeorge syndrome is associated with microdeletions involving chromosome 22q11.2. It is linked to the velocardiofacial syndrome in this same region with 22q11 spanning 3000 kilobases (kb). The microdeletions mediated by homologous recombination between low-copy repeated sequences is detected in 1 in 2000–4000 live births [18,19].

TRISOMY-21 Trisomy-21 or Down's syndrome is associ-ated with thyroiditis and diabetes mellitus in addition to the other classical findings. These patients also have T-cell abnormalities [20].

POEMS SYNDROME Patients with POEMS syndrome (plasma cell dyscrasia with polyneuropathy, organomegaly, endocrinopathy, in protein, in plasma, and skin changes) also have diabetes mellitus, gonadal failure, and neuropathy [21–23].

Patients usually have sensorimotor polyneuropathy, lymphadenopathy, hepatosplenomegaly with plasma cell dyscrasia, and sclerotic bone lesions. The diabetes usually responds to insulin therapy.

CONGENITAL RUBELLA Patients with congenital rubella may develop diabetes mellitus, thyroiditis, and hypothyroidism [24,25]. The diabetes mellitus is associated with *HLA-DR3* and *HLA-DR4* alleles.

ANTI-INSULIN RECEPTOR ANTIBODIES In this rare disorder, which is also known as type B insulin resistance and acanthosis nigricans, the insulin resistance is secondary to anti-insulin receptor antibodies [26,27]. Some patients may also develop other autoimmune diseases such as Sjögren's syndrome and systemic lupus erythematosus.

SUMMARY

The majority of PGAs belong to the PGA II group. Although the gene for PGA I has been cloned and characterized, this has not yet been accomplished for PGA II, but various HLA loci have been implicated in susceptibility to PGA II.

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21 Surgical Management of Endocrine Disorders

GEOFFREY B. THOMPSON, MD

INTRODUCTION

The 20th century saw rapid advances in the surgical management of endocrine disease. High cure rates and low perioperative morbidity have resulted from an emphasis on specialized training and new techniques, as well as improvements in diagnosis, imaging, and surgical pathology.

This chapter focuses on the surgical management of endocrine disorders involving the thyroid, parathyroid, adrenal, and pituitary glands, as well as the gastroenteropancreatic system, which are all subject to the development of endocrine tumors and hyperplasia.

THYROID

The indications for thyroidectomy include suspicion of or biopsy-proven malignancy, compressive symptoms, hyperthyroidism, and rarely cosmetic concerns alone. Nodular thyroid disease is far and away the most common consultation seen by the endocrine surgeon.

Clinically evident, solitary thyroid nodules are found in 4–7% of the U.S. adult population. Upwards of 50% of adults have demonstrable thyroid nodules when ultrasonography or autopsy studies are considered [1]. Most important, however, is that fewer than 5% of thyroid nodules harbor malignancy [2]. A hard, fixed mass with associated cervical lymphadenopathy, rapid growth, young age, hoarseness, and a remote history of neck irradiation are all concerns raising the risk of thyroid malignancy. Fine-needle aspiration biopsy (FNAB) is the most sensitive and specific preoperative indicator of thyroid malignancy. Upwards of 60% of all FNABs will reveal benign cytology, thus avoiding surgical intervention unless one or more of the other described indications are present. Benign cytologies typically include colloid nodules and chronic lymphocytic thyroiditis. Five percent of thyroid nodules will clearly be malignant (papillary or medullary thyroid carcinoma, anaplastic thyroid carcinoma, or metastases). Twenty percent of FNABs are considered suspicious either for follicular or Hürthle cell neoplasms or papillary thyroid carcinoma [3]. Surgery is recommended for the suspicious group, in most instances, because of a 15–20% risk of malignancy in this subgroup. Ten to twenty percent of FNABs are indeterminate or nondiagnostic due to an inadequate or acellular sample. This number can be reduced to below 10%, in experienced hands, by obtaining multiple samples and by utilizing ultrasound-guided and core biopsy techniques for subtle, deep-seated nodules.

Solitary toxic adenomas are best treated with complete thyroid lobectomy [4]. Although radioiodine ablation is a reasonable alternative, especially in patients at high risk for anesthesia, lobectomy has the advantage of eliminating, not just reducing, the size of the nodule as well as maintaining a euthyroid state in a higher percentage of patients. Toxic multinodular goiters (Plummer's disease) can also be treated with radioiodine, but ablation may be difficult if iodine uptake by the gland is low (< 30%) or if the gland is large. In this setting, total lobectomy, isthmectomy, and contralateral near-total lobectomy (near-total thyroidectomy) is the operative procedure of choice, to avoid recurrence, to correct rapidly the hyperthyroid state, and to reduce the risk of permanent hypoparathyroidism (< 3%) [5].

Most cases of Graves' disease are successfully treated in this country with radioiodine. Numerous studies have documented both the safety and efficacy of radioiodine in this setting [6]. A near-total thyroidectomy, leaving a tiny remnant of thyroid tissue to maintain absolute viability of at least one parathyroid gland, is a good option when (1) the gland is large; (2) coexistence of a suspicious or malignant thyroid nodule is present (5%); (3) desire for conception is imminent; (4) rapid correction of the hypermetabolic state is critical (a child with severe behavioral problems or the patient with atrial fibrillation and worsening myocardial function); or (5) children, parents, or adults with Graves' disease voice ongoing fears about the potential long-term effects of radiation exposure.

Patients with autoimmune thyroiditis (both Graves' disease and Hashimoto's thyroiditis) pose significant technical challenges to the endocrine surgeon. The glands are firm, rigid, and highly vascular. The perithyroidal soft tissues tend to be inflamed and thickened, and lymphadenopathy can make identification and preservation of parathyroid glands and recurrent laryngeal nerves a formidable task. Surgical procedures for Graves' disease should be performed only at high-volume centers after a thorough discussion of the various treatment options. In experienced hands,

From: *Endocrine Pathology: Differential Diagnosis and Molecular Advances* edited by Ricardo V. Lloyd © Humana Press Inc., Totowa, NJ.

a near-total thyroidectomy can be carried out for Graves' disease with low morbidity, similar to that of other thyroid operations. In recent years, bilateral subtotal thyroidectomy (leaving 2–3 g of thyroid on each side of the trachea) has been abandoned because of tremendous variability in rates of recurrent hyperthyroidism. With the advent of sensitive thyroid-stimulating hormone (TSH) assays, accurate thyroid hormone replacement has become simple and reproducible.

Rarely are endocrine surgeons asked to perform thyroidectomy for chronic lymphocytic thyroiditis. Most patients are managed effectively with thyroid hormone replacement and normalization of sensitive TSH levels. In some instances, however, large goiters can develop. These can be symptomatic, with symptoms ranging from tightness in the neck, to dysphagia, or rarely intense pain and tenderness. In this setting, near-total thyroidectomy is a reasonable treatment option. Sudden, rapid growth of a known lymphocytic thyroiditis gland can be a harbinger of a non-Hodgkin's B-cell lymphoma. Stage I thyroid lymphomas (confined to the gland) can be managed effectively with total thyroidectomy. Most thyroid lymphomas, however, involve nodes or distant sites and are best managed with multiagent chemotherapy and external beam radiation [7]. Open biopsies or lobectomies may be indicated to establish a definitive diagnosis.

Less common indications for thyroidectomy include amiodarone-induced thyrotoxicosis (when patients with intractable arrhythmias are unable to use alternative medications) and metastatic malignant struma ovarii, to facilitate adjuvant treatment with radioiodine by eliminating the avid uptake of iodine by normal thyroid tissue. Riedel's stroma may require open biopsies to establish this difficult diagnosis. Thyroidectomy should be avoided whenever possible because of the risk of injury to surrounding structures amid the dense fibrotic reaction.

Metastatic tumors to the thyroid gland are rarely isolated to the thyroid. Primary sites most often include the breast, lung, kidney, and skin (melanoma) [8]. Flurodeoxy glucose positron emission tomography (FDG-PET) whole body scanning should be performed along with other conventional imaging procedures before considering a thyroidectomy, unless the metastasis is interfering with the upper aerodigestive tree or causes undue discomfort. Locally advanced tumors, with direct invasion of the trachea or esophagus, are probably best treated with external radiation with or without chemotherapy rather than submit the patient to a formidable resection when life expectancy is likely limited.

Primary thyroid malignancies account for the majority of all endocrine malignancies and endocrine cancer deaths seen by endocrinologists and endocrine surgeons in the United States. Eighteen thousand newly diagnosed cases will occur this year, with slightly more than 1000 cancer-related deaths [9]. Papillary thyroid carcinoma and its follicular variant account for 80–90% of all primary thyroid cancers. Follicular and Hürthle cell cancers comprise 5–10%, medullary thyroid cancer 5%, and anaplastic cancer < 1% [10]. No meaningful progress has been made with regard to anaplastic thyroid cancer. It tends to occur in older patients with sudden, rapid growth in a preexisting, longstanding goiter. Pathology will sometimes reveal areas of differentiated, follicular-cell derived (FCD) cancer among the anaplastic components, suggesting dedifferentiation of an existing FCD cancer, perhaps via mutations in the *p53* tumor suppressor gene mechanism [11]. Despite multimodality therapy with external radiation, surgical debulking, and systemic chemotherapy, most patients die within 12–18 mo from asphyxiation and/or distant metastases. Rarely, thyroid surgeons can perform an isthmectomy and tracheostomy to maintain the airway for brief periods of time.

Although anaplastic thyroid carcinoma is among the worst solid tumors known to mankind, papillary thyroid carcinoma (PTC) has one of the best prognoses. Fortunately, PTC accounts for nearly 85% of all new thyroid cancers. Eighty to eighty-five percent of these papillary cancers can be classified as low risk, associated with a 20-yr cause-specific mortality of <1% with proper treatment [12].

What is meant by low-risk PTC? Review of data, from more than 2000 patients, subjected to a multivariate analysis, at the Mayo Clinic since 1945, have demonstrated that several variables can collectively determine the risk of dying from papillary thyroid cancer. These include: distant **M**etastases, **A**ge (>45), **C**ompleteness of resection (yes or no), **I**nvasion of contiguous structures (trachea, strap muscles, nerve, esophagus), and **S**ize of tumor (>4 cm). This is the **MACIS** system, as defined by Hay et al. [13] and confirmed by other similar scoring systems at institutions worldwide. A simple numerical formula is available for determining each individual MACIS score. A MACIS score of <6 is associated with an excellent prognosis, provided the patient receives appropriate first-line surgical ablation. Preoperative evaluation for most asymptomatic thyroid cancer patients includes a FNAB, a chest X-ray, and a sensitive TSH level. Radioiodine thyroid scans for preoperative evaluation (in euthyroid and hypothyroid patients) are of little value. Ninety-eight percent of "hot" nodules are benign, but so are 92% of "cold" nodules. Neck ultrasonography is, however, useful for both papillary and medullary thyroid carcinoma in detecting clinically occult lymph node metastases.

Ultrasound can help decide whether a central compartment node dissection alone will suffice or whether a unilateral or bilateral modified neck dissections are indicated (Fig. 1). Central compartment nodes include the pretracheal (Delphian), paratracheal (recurrent laryngeal nerve nodes), and the upper anterior–superior mediastinal nodes. A modified or functional neck dissection includes those nodes along the internal jugular vein from the level of the clavicle to the anterior belly of the digastric muscle, as well as those nodes within the posterior triangle. Rarely, suprahyoid nodes are involved. In addition to the carotid artery and jugular veins, important structures at risk for injury or tumor involvement include the following: vagus, phrenic, hypoglossal, and spinal accessory nerves; the sympathetic trunk; cervical and branchial plexuses; as well as the thoracic duct on the left side. A modified neck dissection differs from a classical radical neck dissection by sparing the sternocleidomastoid muscle and the spinal accessory nerve.

Lymph node metastases are an interesting curiosity in low-risk papillary thyroid carcinoma. They seem to affect the local recurrence rate within other nodes, but bear little weight in terms of long-term cause-specific mortality. For PTC, the surgical treatment should include total lobectomy and isthmectomy on the side of the tumor, and a near-total resection on the contralat-

Figure 1 Lymph nodes removed during a central compartment node dissection and a modified neck dissection. (Reprinted from Musholt TJ, Moley JF: Management of persistent or recurrent medullary thyroid carcinoma. *Problems in General Surgery* 1997;14:89–109, Lippincott, Williams & Wilkins, with permission.)

eral side, to reduce the risk of contralateral lobe recurrence. This is seven times more likely to occur when lobectomy alone is performed [14]. Total thyroidectomy should be performed when PTC is grossly bilobar or multicentric (30%). Central compartment nodes should always be removed and lateral nodes should be removed when they are grossly or ultrasonographically involved. Devitalized parathyroid glands should be autotransplanted into the sternocleidomastoid muscle as multiple, 1-mm, implants. These will usually regain function in 4–6 wk time [15].

Little data exist to support the routine use of radioiodine remnant ablation in patients with low-risk PTC who have undergone the operation described. Many endocrinologists, however, still routinely ablate thyroid remnants postoperatively to follow up with whole-body radioiodine scans and thyroglobulin levels (a sensitive tumor marker for FCD tumors). In reality, this is rarely necessary in this group of patients because high-resolution neck ultrasonography and TSH-suppressed thyroglobulin levels will normally suffice [16].

Patients with FCD thyroid cancer may benefit from life-long TSH suppression with thyroxine to reduce the risk of local recurrence. This is reasonably safe as long as sensitive TSH levels are monitored. In this setting, cardiac and bone problems can be kept to a minimum from overzealous treatment with L-thyroxine [17].

Fifteen percent of patients with papillary thyroid carcinoma fall into the high-risk category. It is within this group that the majority of thyroid cancer-related deaths occur. These are the patients with MACIS scores >6 (older age and large, invasive tumors). These also include tumors that are incompletely resected and those with more aggressive histologic subtypes (e.g., tall cell, columnar cell). These patients should undergo as close to a total thyroidectomy as possible (while maintaining parathyroid function), routine central compartment node dissection, and lateral neck dissection when lymph nodes are clinically involved. Radioiodine ablation, therapeutic radioiodine, and maximum TSH suppression with thyroxine (T_4) are essential for these high-risk patients.

The steady decline in both follicular and anaplastic thyroid cancers in the United States has paralleled the decrease in endemic goiter. Most follicular and Hürthle cell neoplasms by FNAB turn out to be adenomas on frozen and permanent sections. Frozen-section evaluation of follicular neoplasms is highly accurate at the Mayo Clinic [18] and reduces the need for a second operation (completion thyroidectomy) in most cases. In a recent review of more than 1000 follicular and Hürthle cell neoplasms, only 8% were found to be malignant on permanent section. Most follicular and Hürthle cell carcinomas today are of the minimally invasive type; gross vascular and capsular invasion are fortunately rare. In patients with known or suspected carcinomas at surgery, a near-total or total thyroidectomy should be performed. Hürthle cell carcinoma patients should also routinely undergo a central compartment node dissection because of a 30% risk of nodal involvement. Patients with follicular car-

cinomas should at least have lymph nodes sampled in the central compartment in case permanent sections demonstrate the follicular variant of papillary thyroid carcinoma. Lobectomy alone may be sufficient for small (< 2 cm), minimally invasive follicular cancers in young people, provided the contralateral lobe is ultrasonographically normal [19]. Widely invasive and Hürthle cell cancers require aggressive adjuvant treatment and follow-up, similar to patients with high-risk PTC. When contiguous structures are truly invaded by PTC or follicular thyroid carcinomas, resection and reconstruction of these structures provides the best chance for cure and local control. Both adjuvant radioiodine and external beam radiation therapy should be employed selectively. Hürthle cell cancers tend not to take up radioiodine.

Medullary thyroid carcinoma (MTC) arises from the thyroid C cells and is therefore unresponsive to radioiodine therapy. Total thyroidectomy, central compartment node dissection, and unilateral or bilateral modified neck dissection are required for proper treatment. Lymph node involvement in MTC is common and, unlike in low-risk PTC, has prognostic significance. Eighty percent of MTC cases are sporadic and are usually unilateral. However, when operating on such patients, one cannot be sure that the patient in question is not an index case for a new multiple endocrine neoplasia type 2 (MEN 2) family; therefore, the need for total thyroidectomy in most cases. Twenty percent of MTC cases are clearly familial and can be confirmed with genetic screening for the *RET* protooncogene mutation associated with MEN 2A, MEN 2B, and familial, non-MEN, MTC. Virtually all familial cases are multicentric. Positive genetic testing today warrants total thyroidectomy in infancy for MEN 2B patients and in early childhood for all others [20]. Biochemical screening for pheochromocytomas must be performed in all MTC patients preoperatively, and, if positive, imaging studies should be obtained (computed tomography [CT], magnetic resonance imaging [MRI], meta-iodobenzyl guanidine I-123 [MIBG scanning], or octreoscanning). Documented chromaffin tumors should be removed (usually laparoscopically) after appropriate pharmacologic blockade and prior to thyroid surgery, to avoid hypertensive crises intraoperatively.

Thyroid surgery boasts an excellent safety record. Most thyroid operations today should be performed with a <1% risk of permanent hoarseness, a 2–3% risk of permanent hypoparathyroidism, and a <0.5% risk of postoperative bleeding (requiring reexploration), and infection. Complication rates will be higher for aggressive malignancies but are acceptable nonetheless, given the life-threatening nature of the disease. Nerve injuries can be managed with thyroplasties, speech therapy, and occasional nerve grafting. Most cases of hypoparathyroidism are easily managed with calcium and vitamin D replacement.

The era of minimally invasive surgery has prompted some thyroid surgeons to utilize laparoscopic technology in an attempt to improve outcomes with both thyroid and parathyroid operations. Unlike abdominal and thoracic procedures where endoscopic surgery has minimized the conventional large, painful muscle-cutting incisions that result in prolonged pain and convalescence, most thyroid and parathyroid operations have for years been carried out through small, muscle-splitting operations just deep to the skin's surface. These heal well, and patients generally return to normal within 7–14 d. Endoscopic neck surgery may provide slight improvement in cosmesis over conventional surgery, but this has yet to be proven in controlled, scientific studies [21].

HYPERPARATHYROIDISM

Primary hyperparathyroidism (HPT) affects 1 in 1000 men and 1 in 500 women in the United States. It occurs when one or more of the parathyroid glands become enlarged in the absence of a secondary stimulus, with resultant hypersecretion of parathyroid hormone (PTH). HPT results in bone resorption, hypercalcemia, hypophosphatemia, and hypercalciuria. Osteoporosis and nephrolithiasis are the two most important clinical manifestations of this disease, but symptoms may be subtle, including fatigue, depression, musculoskeletal complaints, cognitive changes, as well as aggravation of cardiovascular disease. Few, if any, patients are truly asymptomatic, and observation is rarely warranted given the benefit, success, and low risk of parathyroid surgery when performed by experienced endocrine surgeons [22].

Most cases of sporadic HPT involve single adenomas (85–90%). Fewer than 2% of these adenomas are located deep within the mediastinum, necessitating a thoracic approach (median sternotomy, thoracotomy, or thoracoscopy). Ectopic inferior parathyroid glands follow the anterior position of the thymus gland (both third branchial pouch derivatives), and ectopic superior glands follow the tract of the fourth branchial pouch along the tracheoesophageal groove, the retroesophageal region, and finally down into the posterior mediastinum. Other ectopic sites include the carotid sheath, beneath the thyroid capsule, at the level of the carotid bifurcation (undescended inferior gland), and within the pharyngeal musculature. Ten to fifteen percent of sporadic cases are multiglandular, including double adenomas (3–5%). Carcinoma accounts for <1% of all cases. More than 95% of sporadic patients will be cured at the first operation, if performed properly (standard four-gland exploration). Complication rates are similar to those of thyroid surgery. Single-gland disease is treated with excision of the adenoma and the contiguous compressed rim of normal parathyroid tissue. Multigland disease is treated with a subtotal parathyroidectomy, leaving behind a well-vascularized 50-mg remnant. Total parathyroidectomy with immediate autotransplantation in the forearm musculature or chest wall fat is another option, although the risk of permanent hypoparathyroidism with this approach is greater. Patients with the MEN 1 syndrome and familial HPT generally have multigland disease and require a subtotal parathyroidectomy, as well as a transcervical thymectomy; the latter because of the increased risk of a supernumerary gland in these subsets of patients. MEN 2A patients less often develop HPT (<30%), and usually only one or two glands are involved. Because sequelae are less severe in MEN 2A patients, only the visibly affected gland or glands need be removed.

Reoperations for HPT result from inadequate first-time operations, in patients with true mediastinal tumors, in those with multigland disease, and in those with recurrent carcinomas. The success rate of reoperations for benign disease is about 88%, but permanent hypoparathyroidism occurs in as many as 15%, stressing the importance of a thorough first-time exploration and avoidance of removal or damage to normal parathyroid tissue [23].

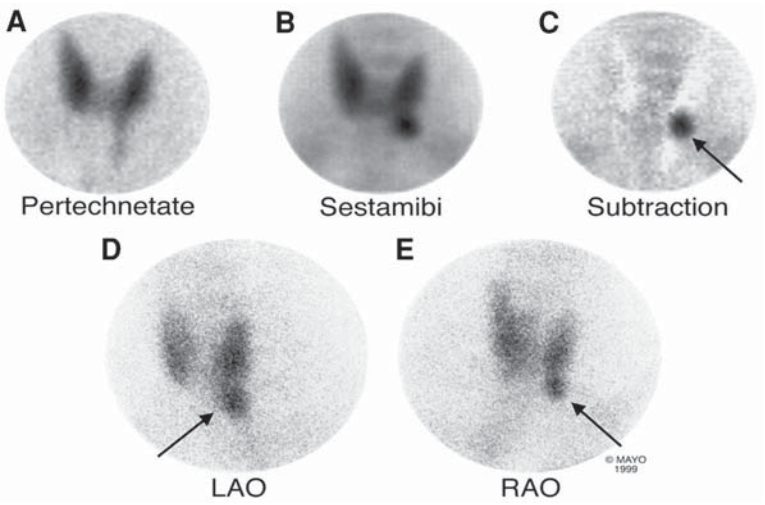

Figure 2 Sestamibi parathyroid scan demonstrating a left inferior parathyroid adenoma (*arrow*).

Parathyroid carcinomas are fortunately rare. They are quite aggressive and often fatal many years after diagnosis. Death usually results from the sequelae of uncontrolled hypercalcemia [24]. Initial treatment involves parathyroidectomy with en bloc resection of the ipsilateral thyroid lobe, lymph nodes, and occasionally the recurrent laryngeal nerve. External beam radiation therapy has been applied in the adjuvant setting and in advanced disease with limited success. Medical therapy includes saline hydration, the use of loop diuretics, and bisphosphates to lower serum calcium levels.

With the advent of sensitive nuclear medicine scanning (sestamibi), single parathyroid adenomas can be detected and localized in 80–85% of cases (Fig. 2). This allows the surgeon to perform a minimal access parathyroid procedure (MAP) through a small incision (3 cm), under local anesthesia, in the outpatient setting (Fig. 3). The dissection is limited and is directed by the scan. The abnormal gland is removed, and rapid intraoperative PTH levels are measured. A >50% drop in PTH from the baseline level at 10 min post-resection is indicative of cure. False positive and negative studies occur, but rarely. If PTH levels do not drop, formal cervical (four-gland) exploration is performed immediately under general anesthesia in a traditional fashion. Since 1998, more than 350 minimal access parathyroidectomies have been carried out successfully at our institution. These procedures have been associated with shorter hospital stays, less pain, less nausea, and perhaps better cosmesis, in many cases. Some surgeons have advocated the use of an intraoperative radio-guided probe, for these procedures, following the intravenous injection of sestamibi. In most centers, this methodology has proven less accurate than the procedures described above [25].

Renal failure and vitamin D deficiency states (rickets, osteomalacia) are the two most common causes of secondary HPT. If left untreated or poorly managed, severe secondary HPT can result, as manifested by severe bone disease, bone pain, fractures, pruritus, and skin ulcerations. When uncontrolled, subtotal parathyroidectomy, or total parathyroidectomy with autotransplantation, may become necessary [26].

In some chronic renal failure patients, who undergo aggressive hemodialysis or renal transplantation, one or more of the hyperplastic parathyroid glands may continue to hyperfunction autonomously. This is so-called tertiary HPT. This condition necessitates parathyroidectomy [27].

ADRENAL DISORDERS

Incidentally discovered adrenal masses on CT, ultrasound, or MRI are common. The vast majority are benign and nonfunctioning. The goal is to predict which ones need to be removed and which ones can be safely observed. All patients with adrenal masses >1 cm in size should be screened for hormonal function. Tests include 24-h urine for total metanephrines and fractionated catecholamines to rule out a pheochromocytoma, a plasma aldosterone/renin ratio to rule out hyperaldosteronism, and a 1-mg overnight dexamethasone suppression test to rule out autonomous cortisol hypersecretion. Excessive hormonal production warrants adrenalectomy. Primary adrenocortical carcinomas are rarely <5 cm in size. Therefore, tumors <4 cm can be safely observed unless interval growth is demonstrated or the tumor has an abnormal radiographic phenotype, suggesting the possibility of malignancy or pheochromocytoma. These radiographic features include a heterogeneous appearance with areas of hemorrhage and necrosis, uptake of intravenous contrast, and increased density on MR T2-weighted images. Such findings warrant removal regardless of size and function [28].

A suspected metastasis to the adrenal gland (lung, breast, renal, gastrointestinal, and melanoma) should never be biopsied without first ruling out a pheochromocytoma, so as to avoid hemorrhage, tumor seeding, or a life-threatening hypertensive crisis, should the tumor in question turn out to be a pheochromocytoma.

Today, most benign functioning and nonfunctioning tumors of the adrenal gland measuring <8 cm can be safely removed through the laparoscope [29]. Laparoscopic adrenalectomy has been a tremendous advance with significant reduction in pain,

Figure 3 Minimal access parathyroid procedure with excision of left superior parathyroid adenoma. (Reprinted from Thompson GB: "No frills" image-guided exploration." *Operative Techniques in General Surgery,* Vol. 1:34–48, 1999; WB Saunders, with permission.)

hospital stay, time to return to normal, and improved cosmesis when compared to open posterior and anterior adrenalectomy (Fig. 4).

Pheochromocytomas occur in about four per one million individuals. Most are sporadic and unilateral, but approx 10% occur in association with MEN 2 syndromes, von Hippel–Lindau disease, neurofibromatosis, and isolated familial pheochromocytoma syndromes, where the risk of bilaterality is increased. Malignancy occurs in <10% of sporadic cases, rarely in familial settings, and in 30% or more of extraadrenal tumors (paragangliomas). Symptoms typically occur in the form of "spells" characterized by paroxysmal hypertension, with headaches, panic

Figure 4 Incisions for laparoscopic left adrenalectomy. (Reprinted from Thompson GB: "No frills" image-guided exploration" *Operative Techniques in General Surgery,* Vol. 1(1):34–48, 1999; WB Saunders, with permission.)

attacks, palpitations, sweating, and chest pain. Measurement of urinary catecholamines and their metabolites or plasma metabolites will confirm the diagnosis in 99% of cases. Imaging with CT, MRI, or MIBG nuclear medicine scanning should localize all but the most occult tumors. Laparoscopic adrenalectomy for smaller pheochromocytomas and open anterior adrenalectomy for larger, malignant-appearing tumors are the surgical procedures of choice following adequate preoperative pharmacologic blockade with both α- and β-antagonists. En bloc resection of contiguous structures (kidney, vena cava, liver) may be necessary when malignant pheochromocytomas involve these organs or major vessels. No adjuvant therapy is presently available, although therapeutic ^{131}I MIBG or external beam radiation therapy may provide effective palliation in select patients [30].

Primary aldosteronism is a common cause of secondary hypertension. It is characterized by drug-resistant hypertension. Patients are often on multiple antihypertensive medications. Hypokalemia occurs in two thirds of patients. Profound muscle weakness is another manifestation secondary to the hypokalemia. The diagnosis should be suspected when high plasma aldosterone to renin ratios are present and is confirmed by elevated, nonsuppressible urinary aldosterone levels after salt loading. The adrenal tumors are usually benign and small. If localization is not clear by CT, adrenal venous sampling can discriminate between a unilateral (adenoma) or bilateral (hyperplasia) source of aldosterone excess. The former is treated with laparoscopic adrenalectomy and the latter with potassium-sparing diuretics and soon to be available selective aldosterone receptor antagonists [31].

Cortisol-producing adenomas account for <20% of all cases of endogenous hypercortisolism. The manifestations of hypercortisolism include truncal obesity, moon facies, hirsutism, acne, pigmented striae, hypertension, glucose intolerance, proximal myopathy, bruising, poor wound healing, opportunistic infection, cataracts, menstrual irregularities, depression, and psychosis. Twenty-four-hour urinary-free cortisol levels >300 mg are diagnostic of Cushing's syndrome. The inability to suppress cortisol levels with dexamethasone and undetectable adrenocorticotropic hormone (ACTH) levels confirm an adrenal source of cortisol excess. CT scanning is the best localizing modality. Laparoscopic adrenalectomy is highly successful and curative for benign adenomas. The contralateral adrenal gland remains suppressed, necessitating a perioperative steroid preparation and slow-taper over several weeks to months [32].

Patients with persistent or recurrent Cushing's disease following transsphenoidal surgery benefit from bilateral laparoscopic adrenalectomy. The incidence of Nelson syndrome is not as high as previously described, and the pituitary can be treated with gamma knife stereotactic neurosurgery should persistent tumor progress. Bilateral laparoscopic adrenalectomy facilitates rapid reduction in cortisol levels and is preferred over total hypophysectomy and fractionated external-beam radiation, particularly in women desiring to have children. Bilateral laparoscopic adrenalectomy has the disadvantages of a life-long need for glucocorticoid and mineralocorticoid replacement therapy, as well as the increased risk of Addisonian crises [33].

Cortisol-producing adrenocortical carcinomas, like all adrenocortical carcinomas, have a poor prognosis. Unlike in patients with pituitary Cushing's disease, the onset of symptoms is rapid. Truncal obesity, striae, and moon facies may be absent, while metabolic and psychiatric manifestations dominate the clinical picture. Cortisol-producing adrenocortical carcinomas may also have elevated levels of aldosterone, testosterone, 17-ketogenic steroids, and dehydroepiandrosterone (DHEA)-sulfate. Tumor thrombi as well as pulmonary and bone metastases are common. Surgical resection at an early stage is the only chance for cure. Therapeutic or adjuvant therapy with mitotane is sometimes beneficial but poorly tolerated due to gastrointestinal side effects. This is a derivative of dithiotreitol (DDT) and has inherent adrenolytic activity. About one-half of adrenocortical carcinomas are functional, while the other half present with pain, fever, and weight loss, usually a sign of advanced systemic disease. Partial debulking is rarely beneficial unless hormonal sequelae dominate the clinical picture. There may, however, be benefit in resecting local recurrences or even isolated metastases to lymph nodes or pulmonary sites [34].

Less common causes for ACTH-independent Cushing's disease, requiring bilateral laparoscopic adrenalectomy, include bilateral macronodular adrenocortical hyperplasia and primary pigmented nodular adrenal disease (PPNAD); the latter is seen in Carney complex.

Ten percent of patients with endogenous hypercortisolism have ectopic ACTH syndrome characterized by high 24-h urine cortisol levels and very high plasma ACTH levels. In general, cortisol levels are nonsuppressible with dexamethasone. Tumors of the chest are most often implicated, including small cell cancers of the lung, bronchial carcinoids, and thymic carcinoids. Medullary thyroid carcinomas, islet cell carcinomas, and pheochromocytomas can also produce ACTH- or corticotropin releasing hormone (CRH)-like substances. Treatment is generally directed at the primary tumor, but when occult, unresectable, or metastatic, bilateral laparoscopic adrenalectomy can provide significant palliation from the manifestations of hypercortisolemia [35].

Other rare indications for adrenalectomy include myelolipomas, cysts, and adrenal hemorrhage when the diagnosis is uncertain or pain is an issue.

GASTROENTEROPANCREATIC TUMORS

GASTROINTESTINAL CARCINOID TUMORS Carcinoid tumors occur throughout the body but are most commonly associated with the gastrointestinal tract. Appendiceal carcinoids are the most common and least aggressive. Unless the tumor is >2 cm (a rarity), involves the base of the appendix, or has metastasized to regional nodes, simple appendectomy alone with suffice and is curative; for all others, a right hemicolectomy is required [36].

Small bowel carcinoids occur with increasing frequency from the duodenum to the terminal ileum. Carcinoid tumors are the most common malignant tumors of the ileum. They frequently metastasize, even when small, to regional lymph nodes and later to the liver. When hepatic metastases are large or diffuse, the liver's ability to metabolize serotonin and other bioactive amines can be overwhelmed, leading to the malignant carcinoid syndrome as manifested by crampy diarrhea, flushing, asthma, and right-sided valvular heart disease [37].

Primary treatment of small bowel carcinoids includes small bowel or ileocolonic resection with en bloc resection of the lymph node-bearing mesentery. Bulky nodal metastases can result in bowel obstruction and/or mesenteric ischemia [38]. Similarly to metastatic islet cell tumors, carcinoids, metastatic to the liver, can be treated effectively in a number of ways due to the indolent nature of this tumor. These methods include hepatic artery (chemo) embolization, surgical debulking (hepatectomy and metastasectomy), radiofrequency thermoablation, cryoablation, and more conventional forms of chemotherapy, hormonal therapy (octreotide), and hepatic transplantation [39].

Duodenal carcinoids are frequently gastrin producing and are discussed under the heading of Zollinger-Ellison syndrome. Colonic carcinoids are aggressive, usually nonfunctioning, and are treated with colonic resection, like any colon cancer. Rectal carcinoids are usually small and superficial and are best treated with proctoscopic excision and fulguration when seen. The rare, large, invasive rectal carcinoid is an aggressive tumor managed by low anterior resection or abdominal perineal resection, depending on its proximity to the anal verge [40].

Gastric carcinoids are most often a consequence of achlorhydria (chronic atrophic gastritis) and secondary hypergastrinemia resulting in enterochromaffinlike (ECL)-cell hyperplasia and the formation of multiple tumors. These tumors rarely, if ever, metastasize. Endoscopic surveillance, endoscopic ablation, and symptomatic treatment are all that is usually necessary. Antrectomy has been suggested by some to eliminate the gastrin excess, but its efficacy has never been proven. Indications for surgical excision or gastrectomy include tumors > 2 cm in size, atypical carcinoid histology, as well as the presence of gastrin-secreting or serotonin-producing tumors [41].

ZOLLINGER–ELLISON SYNDROME (ZES) ZES is characterized by gastric acid hypersecretion and hypergastrinemia. ZES can present with a severe ulcer diathesis, gastroesophageal reflux disease, or diarrhea alone. The diagnosis is confirmed by documenting high serum gastrin levels concomitantly with gastric acid hypersecretion off all antisecretory drugs. Most cases are due to a small, gastrin-producing, submucosal duodenal carcinoid tumor. Although malignant, these tumors can be effectively managed with submucosal excision and regional lymphadenectomy. Whipple procedures are reserved for large duodenal or pancreatic head tumors and distal pancreatectomy for body and tail masses. Although recurrence rates as high as 50% have been reported, long-term survival is the rule [42].

In MEN 1 patients, hypergastrinemia is common. Although these patients frequently have multiple islet cell tumors, the source of the gastrin excess is usually confined to multiple duodenal carcinoids. As recent reports have documented that the most common cause-specific mortality in MEN 1 patients are the pancreatic and duodenal tumors, many surgeons have promoted early surgical intervention. The Ann Arbor procedure, popularized by Thompson et al. [43], is the most common operation performed. This includes a distal pancreatectomy and splenectomy, enucleation of pancreatic head and duodenal tumors, as well as a regional lymphadenectomy. Preliminary results suggest that this operation may prevent or delay disease progression. Long-term studies are still being carried out (Fig. 5).

INSULINOMAS Insulinomas occur with a frequency of four per one million person-years. They are usually benign, single, < 2 cm in size, and virtually all are intrapancreatic. Symptoms of neuroglycopenia occur typically during fasting and exercise and are essential to the diagnosis. The 72-h fast forms the basis for diagnosis. Fasting hypoglycemia (< 45 mg/dL) with neuroglycopenic symptoms, elevated proinsulin and C-peptide levels, absent insulin antibodies, and a negative drug screen for sulfonylureas confirm the diagnosis of endogenous hyperinsulinism. Localization is possible in 60–70% of patients preoperatively with transabdominal ultrasound, CT, and endoscopic ultrasonography (Fig. 6). Selective arterial calcium stimulation with hepatic vein sampling for insulin is highly accurate for regionalizing the location of an insulinoma to the head, uncinate, body, or pancreatic tail, but it is quite expensive and invasive. With a secure diagnosis, an experienced endocrine surgeon can locate 98% of all insulinomas with palpation and intraoperative ultrasonography alone. Enucleations are preferred for small pancreatic tumors, while distal pancreatic resections and Whipple procedures are reserved for large or malignant tumors [44]. Laparoscopic pancreatic resection and enucleations are being performed in very select cases.

Some hypoglycemic patients demonstrate severe postprandial neuroglycopenia with a negative 72-h fast. These unique patients may have noninsulinoma pancreatogenous hypoglycemia syndrome (NIPHS), an adult form of nesidioblastosis. Confirmation can be obtained by demonstrating a positive calcium stimulation test with a greater than twofold increase in insulin concentrations in one or more pancreatic arterial distributions. Gradient-guided pancreatic resections can aid in palliating these desperately ill patients [45,46].

MEN 1 patients with hyperinsulinism are treated with an extended distal pancreatectomy to the right of the portal vein because of the multiplicity of tumors and nesidioblastosis.

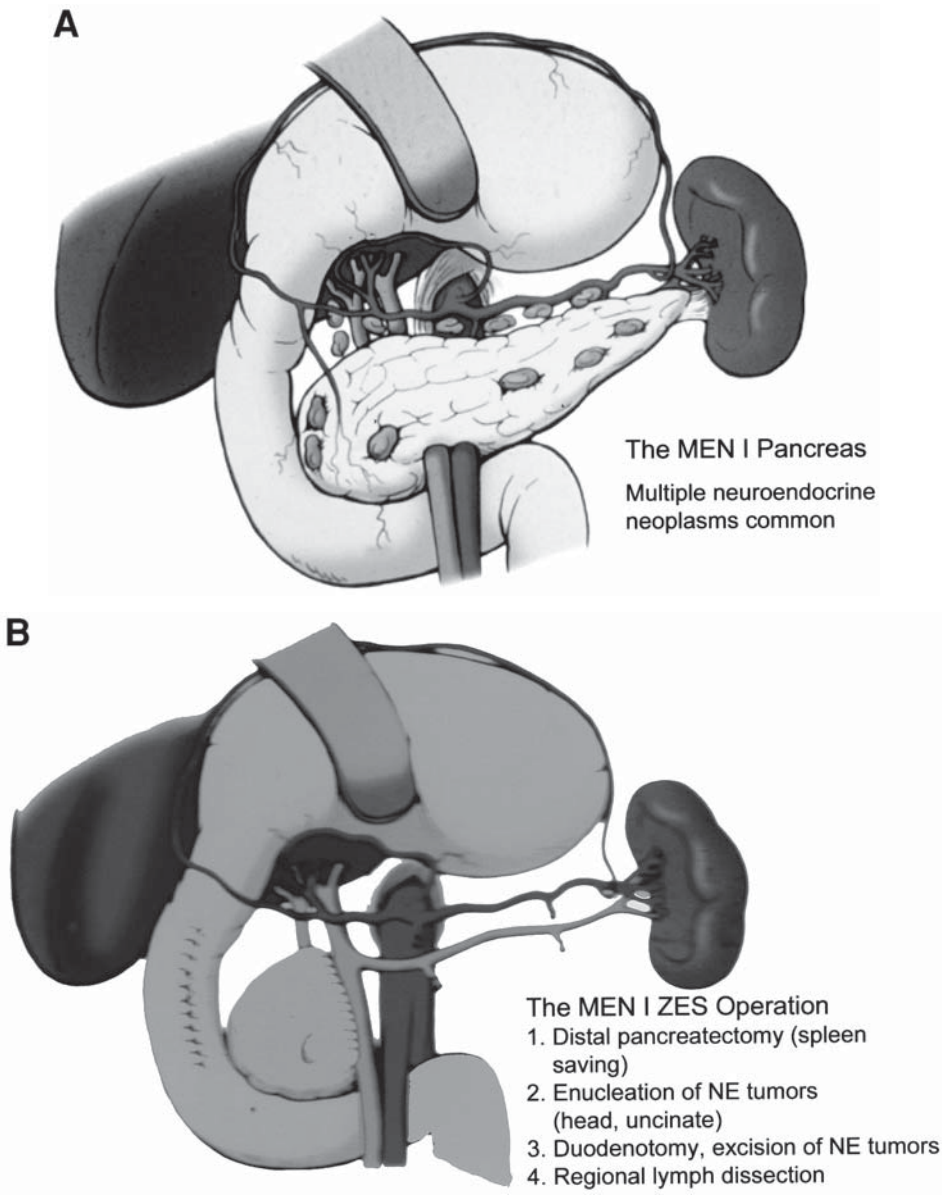

Figure 5 Pancreaticoduodenal procedure for the MEN 1 pancreas. (Courtesy of Dr. Norman Thompson, Ann Arbor, MI.)

GLUCAGONOMAS The glucagonoma syndrome is characterized by diabetes, necrolytic migratory erythema, a large malignant α-cell tumor of the pancreas, and hyperglucagonemia. Metastases are frequent, and cures are rare. Distal pancreatectomy or Whipple procedures are indicated, depending on the tumor's location. Pulmonary emboli are frequent cause of death (Figs. 7A,B) [47].

VASOACTIVE INTESTINAL POLYPEPTIDE-OMA (VIPoma) VIPomas are usually large, malignant, islet cell tumors of the pancreas associated with hypersecretion of VIP. They are less commonly associated with benign ganglioneuromas. The clinical syndrome (Verner–Morrison syndrome) is characterized by profuse secretory diarrhea, hypochlorhydria, hypokalemia, dehydration, acidosis, hypercalcemia, and, if left untreated, renal failure, shock, and fatal arrhythmias. Treatment is similar to that of other malignant islet cell tumors. The long-acting somatostatin analog can be beneficial in controlling fluid and electrolyte abnormalities during the perioperative period [48].

SOMATOSTATINOMAS These are often large neuroendocrine tumors involving the pancreatic head or duodenum. They occur sporadically but also in association with type 1 neurofibromatosis. Somatostatin is a potent inhibitory peptide, and, therefore, the clinical manifestations of diabetes, steatorrhea, gallstones, and achlorhydria. Treatment usually requires pancreatoduodenectomy [49].

LESS COMMON ISLET CELL TUMORS These include tumors that secrete ACTH, corticotrophin-releasing factor (CRF), melanocyte-stimulating hormone (MSH), cholecystokinin (CCK),

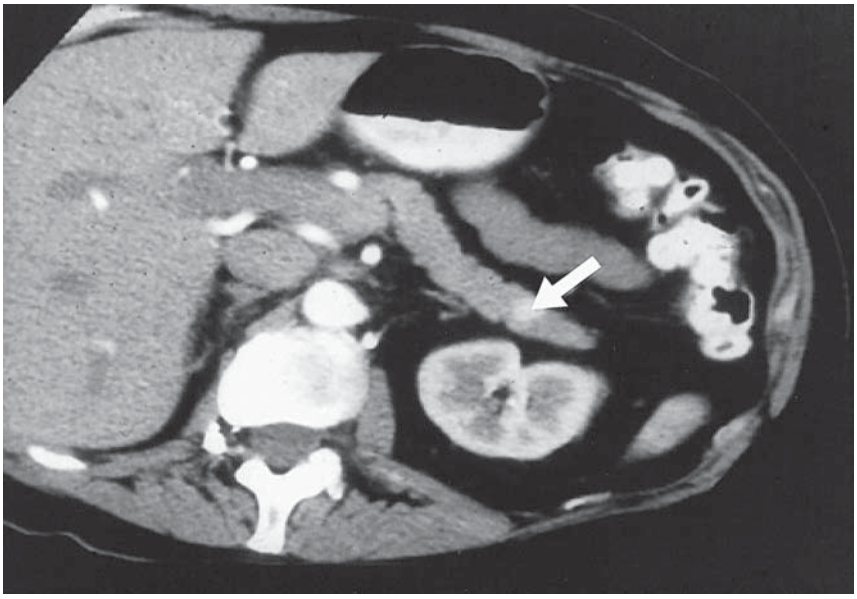

Figure 6 Spiral CT scan demonstrating an insulinoma in the pancreatic tail. (Courtesy of C.C. Reading, Rochester, MN).

serotonin, and pancreatic polypeptide. Most are large and malignant, and are, therefore, managed accordingly.

NONFUNCTIONING ISLET CELL TUMORS These are the most common islet cell tumors. Most are malignant and present with pain, jaundice, steatorrhea, weight loss, and sinistral hypertension from splenic vein thrombosis. Treatment is the same as with other pancreatic malignancies, although the prognosis is better than with pancreatic ductal adenocarcinoma [50].

PITUITARY

PITUITARY ADENOMAS Pituitary adenomas account for 10–15% of all intracranial tumors. Pituitary adenomas present with hormonal sequelae, mass effect (headache, visual disturbances, or hypopituitarism), or both. Pituitary incidentalomas are common and are generally of little clinical significance. Nonsecretory tumors account for 40% of all pituitary neoplasms, and when symptomatic, typically present with mass effects.

PROLACTINOMAS Prolactinomas are the most common functioning pituitary adenomas. In women, they cause galactorrhea–amenorrhea syndrome. In men, prolactinomas may present with decreased libido, impotence, and oligospermia. Most prolactinomas are microadenomas, and dopamine agonists are the mainstay of treatment. Indications for transsphenoidal surgery include intolerance or unwillingness to take dopamine agonists, desire for pregnancy, or mass effect. Surgery is associated with a 70% success rate, long term [51].

CUSHING'S DISEASE ACTH-dependent Cushing's disease of pituitary origin is characterized by the slow onset of signs and symptoms of Cushing's syndrome. Urinary-free cortisol levels are often intermittently elevated due to cyclical secretion of cortisol. ACTH levels may be only mildly elevated. A positive CRH-stimulated, low-dose dexamethasone suppression test is confirmatory. MRI detects approx 60% of ACTH-secreting tumors. When in doubt, petrosal sinus sampling with CRH stimulation can confirm the pituitary origin of the syndrome, as well as lateralize the tumor appropriately. Endoscopic transsphenoidal surgery via an endonasal approach is now being performed on a regular basis with faster recovery and less facial pain (Fig. 8) [52]. Transsphenoidal surgery is associated with 65–85% long-term cure rates in patients with Cushing's disease [53].

GROWTH HORMONE SECRETING TUMORS Acromegaly is a clinical syndrome that results from excess growth hormone due to a benign pituitary tumor in 99% of cases. Morbidity and mortality rates are increased in untreated acromegalics. In addition, mortality rates from colon and breast cancer are increased. Microadenomas have a cure rate of 91% and macroadenomas 48% following transsphenoidal surgery. Growth hormone and insulinlike growth factor (IGF)-1 levels can also be decreased with conventional fractionated radiotherapy, gamma knife stereotactic neurosurgery, somatostatin derivatives, and dopamine agonists. Surgery remains first-line therapy in most cases, especially when mass effect is present [54].

SURGERY FOR DIABETES

Much work remains to be done with regard to islet cell transplantation for type 1 diabetes. This topic is beyond the scope of this chapter. Whole organ transplantation, particularly when combined with kidney transplants in type 1 diabetics, has met with increasing success. Long-term graft survival, however, has yet to approach that of the kidney, liver, or heart transplants. Patients with medically complicated morbid obesity and type 2 diabetes are achieving dramatic results and cures with combined restrictive and malabsorptive procedures (Roux-Y gastric bypass [Fig. 9]) that facilitate and maintain significant weight loss (> 50% of excess body weight) [55].

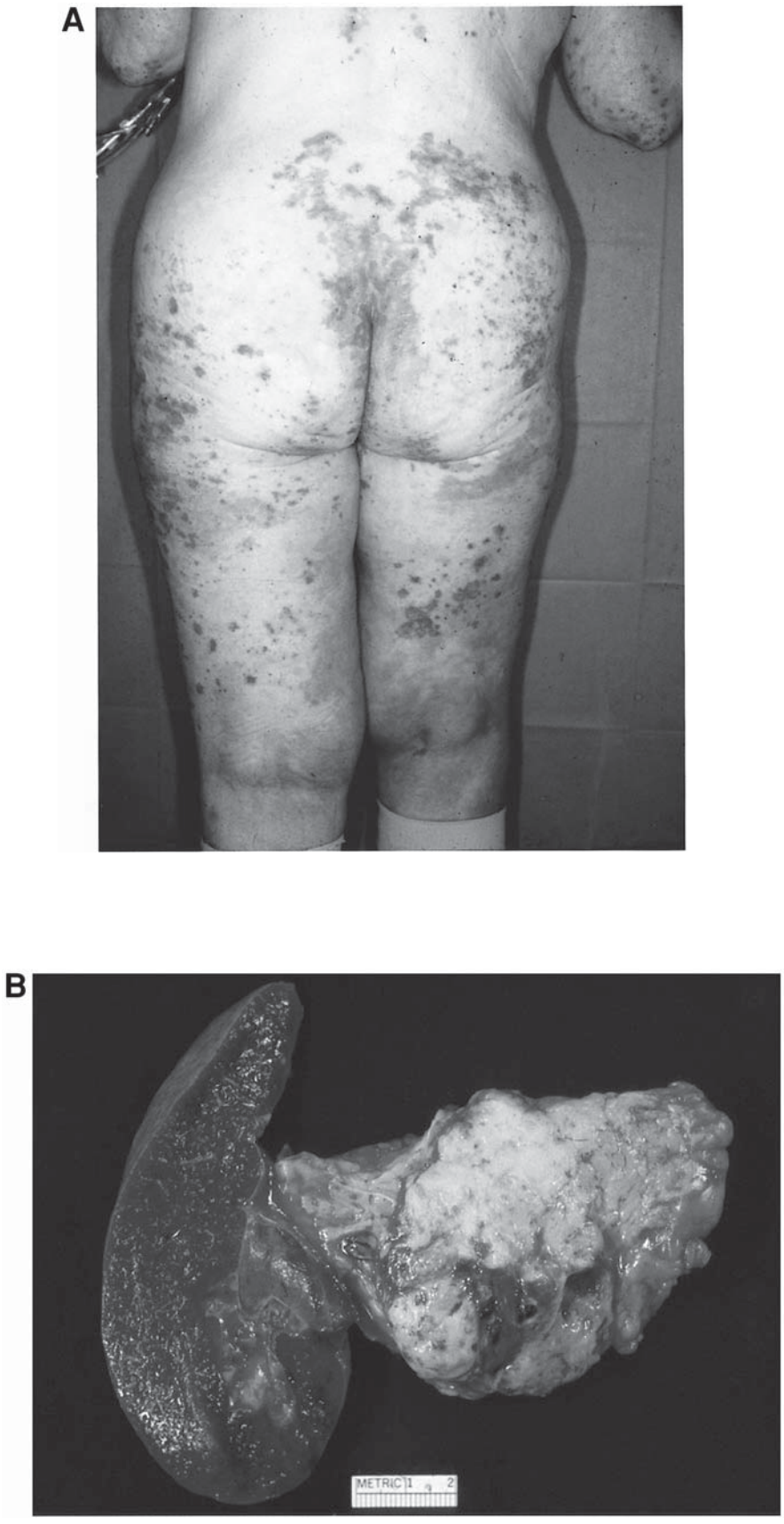

Figure 7 (**A**) Necrolytic migratory erythema. (**B**) Distal pancreatectomy specimen from the patient in **A** with a malignant glucagonoma (rash resolved completely after resection).

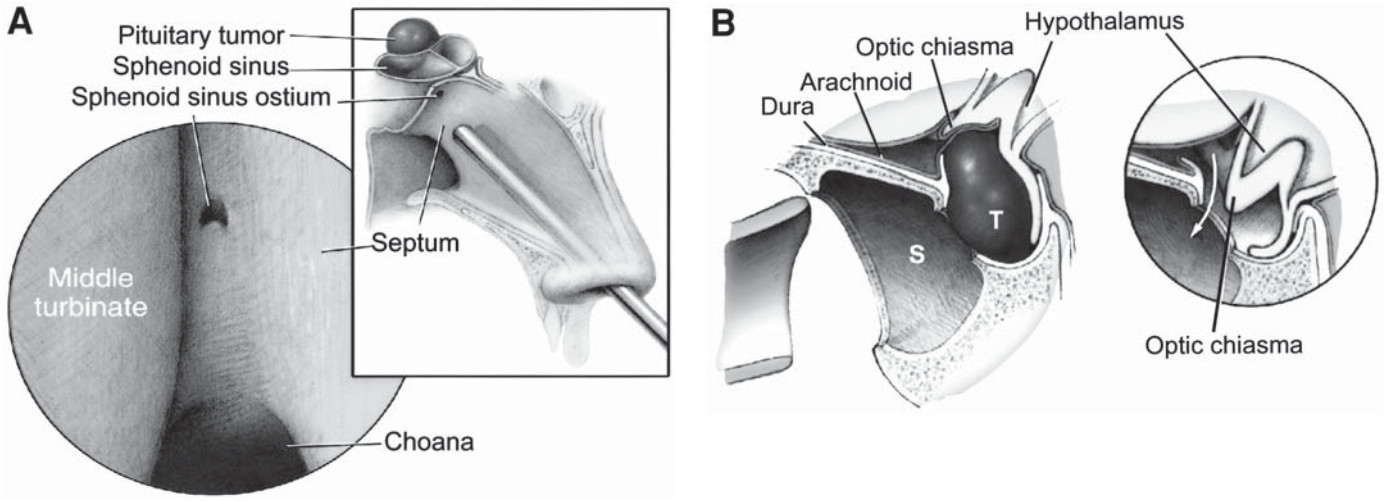

Figure 8 Endoscopic transsphenoidal pituitary surgery. (Courtesy of Dr. John Atkinson, Rochester, MN.)

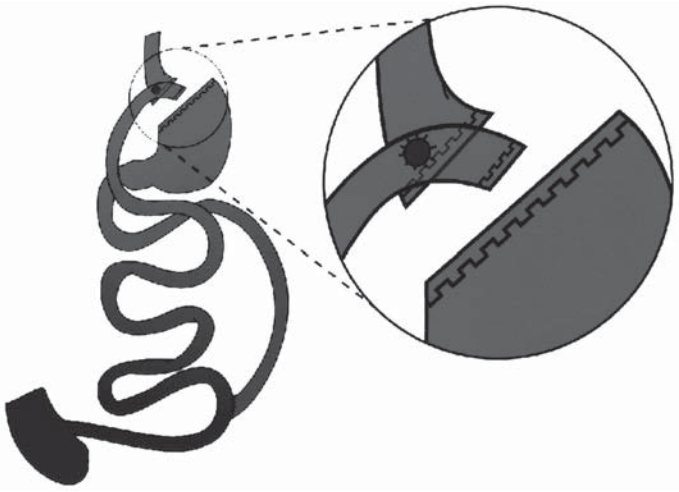

Figure 9 Roux-Y gastric bypass procedure for medically complicated obesity.

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22 Medical Treatment of Advanced Neuroendocrine Tumors

TIMOTHY J. HOBDAY, MD AND JOSEPH RUBIN, MD

INTRODUCTION

The neuroendocrine tumors (carcinoid tumor, islet cell carcinoma, and other neuroendocrine neoplasms) present a unique challenge to the practice of medical oncology. The clinical heterogeneity of these patients and the variable natural history of these tumors demand a careful, individualized approach to each patient. One must consider the histology and location of the patient's primary and metastatic tumors, the symptoms due to the tumor bulk itself, the symptoms due to the overproduction of various hormones and peptides, and the multidisciplinary options available for curative and palliative intent therapy. Many patients initially present with distant, even bulky, metastatic disease that is asymptomatic or minimally symptomatic. For these patients, it may be appropriate to consider no antitumor therapy at all as an initial strategy. The possibility of prolonged stable disease with preserved quality of life is real. This approach can spare some patients the potential toxicity of palliative-intent therapy for years.

CLINICAL CONSIDERATIONS

METASTATIC CARCINOID TUMORS Carcinoid tumors may arise from virtually any organ; however, most are derived from foregut (pulmonary, gastric), midgut (small intestine, appendix), and hindgut (rectum). More than 95% of carcinoids arise from the appendix, small intestine, and rectum. The metastatic potential is related to size, with carcinoids of the appendix and rectum virtually always cured if the primary tumor is <2 cm and 1.5 cm, respectively. Smaller carcinoids of the small intestine may show more metastatic potential [1]. Several clinical syndromes are important in the management of metastatic carcinoids.

Carcinoid Syndrome The classic carcinoid syndrome consists primarily of flushing and diarrhea, each seen in about three-fourths of patients with the carcinoid syndrome. Wheezing is much less common. The syndrome is thought to be associated with the overproduction of serotonin, which is commonly measured as its urinary metabolite 5-hydroxyindoleacetic acid (5-HIAA). However, some patients with elevated urinary 5-HIAA levels have few or no symptoms, and other substances have been postulated to play a role in the development of the syndrome, such as prostaglandins, bradykinins, histamine, and gastrin. The carcinoid syndrome generally suggests advanced disease with tumor access to the systemic circulation, usually through liver metastases. However, primary ovarian and testicular carcinoids can cause the syndrome through direct venous drainage to the caval circulation. Rectal carcinoids rarely, if ever, produce serotonin and the carcinoid syndrome. Flushing can be precipitated by many stimuli that presumably alter vascular tone such as emotional and physical stress, exertion, and alcohol [1]. In its extreme form, the carcinoid crisis can occur, often with anesthesia, surgery, hepatic artery embolization, and, rarely, spontaneously. Carcinoid crisis results in extreme hemodynamic instability with severe hypertension or hypotension. Prompt therapy with somatostatin analogues can be life saving [2].

Mesenteric Masses Regional metastases to the mesentery and retroperitoneal lymph nodes are common in patients with small intestinal carcinoids. Mesenteric metastases can cause an intense fibrotic reaction that can result in tethering of the mesenteric border of the bowel and the propensity to small bowel obstruction. Masses at the root of the mesentery and regional lymph nodes can obstruct the arterial and venous circulation of the intestine causing ischemic symptoms. Surgical resection may be the treatment of choice when feasible. Antitumor therapy to prevent the onset of these symptoms may be considered in select patients.

Carcinoid Heart Disease Carcinoid heart disease is another distinct complication of overproduction of serotonin, generally for several years' duration, and suggests advanced disease. Tricuspid and pulmonic valve regurgitation and stenosis result from smooth, pearly plaques that deposit on the endocardium and subendocardium. Surgical valve replacement or repair can be considered at experienced centers, if control of the systemic disease is adequate. The use of echocardiography can allow the identification of patients with early cardiac involvement. This may prompt more aggressive therapy in hopes of preventing or delaying the progression to overt heart disease [3].

ISLET CELL CARCINOMAS AND OTHER NEUROENDOCRINE CARCINOMAS Advanced islet cell carcinomas and other neuroendocrine carcinomas can present either through local symptoms (such as obstructive jaundice, pain, or steator-

From: *Endocrine Pathology: Differential Diagnosis and Molecular Advances* edited by Ricardo V. Lloyd © Humana Press Inc., Totowa, NJ.

rhea), symptomatic and bulky distant metastases, or as one of a myriad of hormonal syndromes. Hormonal syndromes can occur in both localized and distant disease. Excessive production of gastrin can result in diarrhea and the Zollinger–Ellison syndrome of multiple and recurrent peptic ulcers. Overproduction of glucagon can result in diabetes mellitus and a characteristic dermatitis. Insulinomas result in symptomatic hypoglycemia while excessive vasoactive intestinal peptide (VIP) production can result in the syndrome of pancreatic cholera. Many hormones can be less commonly encountered such as serotonin, adrenocorticotropic hormone (ACTH), somatostatin, parathyroid hormone, vasopressin, and human pancreatic polypeptide. A significant minority of patients with islet cell carcinomas can produce multiple different hormones.

It is important to keep in mind that multiple endocrine neoplasia type 1 (MEN 1) can be present in patients with islet cell carcinomas, and a careful medical history, family history, physical examination, and laboratory evaluation of these patients are all important factors to assist in identifying this syndrome. Common features of this syndrome are tumors of the parathyroid and pituitary glands as well as enteropancreatic neuroendocrine tumors. The neuroendocrine tumors are usually islet cell carcinomas, but foregut carcinoids can also be seen. The beneficial effect of identifying MEN 1 for the patient and family members can be enormous, as careful screening and/or DNA testing for a mutation of the putative tumor suppressor gene located on chromosome 11q13 can be offered.

THERAPEUTIC OPTIONS FOR METASTATIC NEUROENDOCRINE TUMORS

SOMATOSTATIN ANALOGS Somatostatin analogs are effective therapy for many patients with symptoms due to hormonal excess, such as the carcinoid syndrome, pancreatic cholera, symptomatic hypoglycemia, or peptic ulcer disease. Side effects are generally mild or nonexistent, consisting most importantly of occasional steatorrhea and a significant incidence of gallstone formation with prolonged use. Symptomatic cholelithiasis is uncommon [4]. For patients with the carcinoid syndrome, treatment success (defined by symptomatic control of the syndrome without the need for other supportive measures) occurred in approx 65% of patients in a recent clinical trial demonstrating the therapeutic equivalence of the short- and long-acting formulations of octreotide acetate [4]. Precise estimates as to the frequency of substantial hormonal response to somatostatin analogs in patients with islet cell carcinomas are hampered by small series in the literature, but likely happen less frequently.

Somatostatin analogs also seem to possess some antiproliferative activity in neuroendocrine tumors. Partial regressions of carcinoid tumors and several other neuroendocrine tumors have been reported. A German multicenter phase II trial of octreotide of 52 patients with gastroenteropancreatic neuroendocrine tumors and documented tumor progression prior to octreotide therapy was conducted. Nineteen patients (36.5%) had stabilization of disease at some point after 3 mo of therapy, 12 patients had disease stability after 1 yr, and nine patients after 2 yr. No tumor regression was seen [5]. Another phase II trial demonstrated a 50% incidence of disease stabilization with octreotide in these patients, but also found no tumor regressions [6]. It appears that carcinoid tumors may be more likely to demonstrate stabilization of growth and rare regressions than other neuroendocrine tumors. However, a recent series of 13 patients with metastatic gastrinomas to the liver and with disease progression documented disease stabilization in 47% of patients with one tumor regression for a total antitumor response rate of 53% [7]. For nonfunctioning neuroendocrine tumors, we favor confirmation of the presence of somatostatin receptors, generally through radionuclide scintigraphy, prior to the use of these drugs when the intent is disease stabilization.

For patients who remain or become symptomatic despite the use of somatostatin analogs or who are not felt likely to benefit from this therapy, more aggressive therapy is needed. Palliative surgical options and other regional strategies should be considered prior to systemic therapy if it is felt that a significant impact on the bulk of the tumor and its attendant symptoms might be obtained. If there are symptoms potentially related to small bowel obstruction and/or ischemia, these patients can often gain significant palliation with surgical resection of local and regional disease, even in the presence of distant disease. There are many patients who have the preponderance of their tumor bulk in the liver. For properly selected patients, severe endocrinopathies or symptoms due to tumor bulk can be promptly relieved with durable palliation if the majority of hepatic disease can be surgically resected [8]. Unfortunately, symptomatic disease will recur with time in most patients, and/or the extent of metastatic disease in the liver may be too diffuse in many patients to address surgically. In these patients, an alternative regional treatment strategy may need to be considered.

HEPATIC ARTERY EMBOLIZATION OR CHEMOEMBOLIZATION Most metastatic tumors to the liver derive more than 80% of their blood supply from the hepatic artery, while normal liver parenchyma is supplied mostly from the portal vein [9]. This differential perfusion allows "selective" treatment of the metastatic disease through surgical ligation or catheter-based embolization of the hepatic artery. Concurrent chemotherapy is also given through the hepatic artery catheter with the embolization at many institutions. Both of these approaches can allow effective and sometimes durable palliation of symptoms due to bulky liver metastases. There has been no direct comparison of "bland" hepatic artery embolization (HAE) to chemoembolization (CE) in these patients to allow for evaluation of the additional benefit and toxicity purported by some for CE. Toxicity from either procedure can be problematic with the typical patient experiencing fever, right upper quadrant pain requiring narcotic analgesia, and fatigue with each procedure. Carcinoid crisis and other "flares" of hormonal syndromes can occur shortly after the procedure. Life-threatening complications of hepatic and/or renal failure, cardiac arrhythmia, and hepatic abscess can occur. The use of "staged" procedures repeated perhaps two or three times at 4–6 wk intervals consisting of selective embolization directed at regions of maximal tumor bulk or limited to one-third or one-half of the liver attempts to allow complete treatment of the hepatic disease bulk with less acute toxicity.

Moertel and colleagues at the Mayo Clinic in the late 1970s and early 1980s reported the first extensive experience for

hepatic artery embolization. They documented 50% of patients with carcinoid tumors, and 71% of patients with islet cell carcinomas had a >75% decline in hormonal levels after a single HAE with corresponding objective regressions, but the median duration of benefit was only 5 mo [1]. This led to a large study of 111 patients comparing HAE alone vs HAE followed by a sequential chemotherapy regimen alternating adriamycin and dacarbazine with streptozotocin and 5-fluorouracil. Regression rates for carcinoid tumors were 65% for HAE alone and 81% for the combination, while the corresponding rates were 53% and 79% for islet cell carcinomas. The duration of regression (6.6 mo vs 20 mo in carcinoid patients and 4 mo v 20 mo in islet cell carcinoma) and overall survival (27 mo vs 49 mo for carcinoid patients and 9 mo vs 35 mo for islet cell carcinoma) for patients treated with the combination were far superior in both disease entities as compared to patients treated with HAE alone. The results of this study, however, may have been influenced by the lack of randomization and the imbalance in important patient characteristics such as prior chemotherapy, duration of hepatic metastases, and performance status. Also, a single HAE was performed as compared to multiple staged procedures currently more common [10].

The addition of hepatic artery infusion of chemotherapy or encapsulated particles containing chemotherapy has been advocated in an attempt to deliver high concentrations of anti-tumor agents to areas of hypoxic tumor. Several different agents such as 5-fluorouracil (5-FU), streptozotocin (STZ), cisplatin (CDDP), and adriamycin (ADR) have been used in small (10–25 patients, generally) single-institution studies. Symptomatic benefit has been suggested in 60–100% of patients, with objective regressions in 33–78%. Patients with the carcinoid syndrome tended to have more frequent symptomatic responses and for a longer duration than those with islet cell carcinomas in these studies [11–14]. Direct comparisons of CE and HAE in terms of toxicity and effectiveness are not possible with existing data.

CHEMOTHERAPY The evaluation of chemotherapy in the neuroendocrine tumors has been limited to small randomized and single-arm clinical trials. The clinical heterogeneity of these patients makes any cross-study comparisons of various agents even more problematic than in most cancers. Also, there has been much variation in the parameters that are used to define response to therapy, be it radiographic, biochemical, or a combination of these factors. In general, chemotherapy has been most beneficial for patients with clinically aggressive and/or high-grade disease or with islet cell carcinomas. Chemotherapy has held little benefit for those with typical carcinoid tumors.

Small case series of patients treated with chemotherapy for metastatic neuroendocrine tumors in the 1970s and 1980s suggested activity of a number of single agents including 5-FU, dacarbazine (DTIC), ADR, cyclophosphamide (CTZ), CDDP, and actinomycin D. The nitrosurea antibiotic STZ was noted preclinically to induce diabetes mellitus through islet cell destruction. This led to clinical trials of this agent for patients with neuroendocrine tumors [15].

Chemotherapy for Carcinoid Tumors The Eastern Cooperative Oncology Group (ECOG) conducted a randomized trial of STZ/5-FU vs STZ/CTZ for patients with carcinoid tumors. There was no difference between the two regimens, and response rates were 33% and 27%, respectively, with median survival of approx 2 yr [16]. A subsequent randomized trial compared STZ/5-FU with ADR in the treatment of carcinoid tumors. Again, response rates were modest (22% and 21%), median survival was about 1 yr, and there was no clear difference between the two regimens. Some patients who progressed on the combination arm crossed over to the ADR arm with an 18% response rate, generally of short duration [17]. A study by the Southwest Oncology Group of a combination of STZ/5-FU/ADR/CTX also documented a response rate of 31%, but a median survival of only 10.8 mo [18]. More recent empiric trials of newer combinations (5-FU/ADR/CDDP) [19] and single agents such as carboplatin [20], taxol [21], and topotecan (J. Rubin, *personal communication*) have not suggested an improvement over prior therapies. At the Mayo Clinic, we strongly recommend participation in clinical trials for patients with symptomatic carcinoid tumors in need of systemic therapy.

Chemotherapy for Islet Cell Carcinoma As opposed to the minimal benefit of systemic chemotherapy for patients with typical carcinoid tumors, a significant percentage of patients with islet cell carcinomas can derive some durable palliative benefit with chemotherapy. In the 1970s, STZ as a single agent was compared with the combination of 5-FU/STZ. The combination demonstrated an improvement in response rate and suggestion of prolonged survival [22]. The reported activity of ADR led to a randomized trial of STZ/ADR vs 5-FU/STZ and a third arm of chlorozotocin. STZ/ADR was superior to the other two arms, with a statistically significant increase in response rate (69% vs 45% for 5-FU/STZ, $p = 0.05$), time to progression (20 mo vs 6.9 mo for 5-FU/STZ, $p = 0.001$), and median survival (2.2 yr vs 1.4 yr, $p = 0.004$) [23]. This remains a standard regimen for islet cell carcinoma.

Chemotherapy for High-Grade and/or Clinically Aggressive Neuroendocrine Tumors Moertel et al. conducted a phase II study of etoposide and cisplatin in 45 patients with metastatic neuroendocrine tumors. The response rate for typical low-grade tumors was only 7%. However, for patients with anaplastic variants of these tumors, the response rate was 67%, with a median duration of regression of 8 mo and a median survival of 19 mo. These appear to be superior outcomes compared with that expected for these more aggressive histologies [24]. Fjallskog et al. recently published their experience with clinically aggressive (although not necessarily high-grade histologically) tumors with a similar regimen. Some had received prior chemotherapy and progressed. Radiographic and/or biochemical responses in 56% of patients with foregut carcinoids, and 7 of 14 patients with islet cell carcinomas were reported [25]. Toxicity, however, can be considerable with this active regimen.

INTERFERON-α Interferon-α has been investigated in the treatment of primarily carcinoid tumors, although there are series and reports of use in islet cell carcinoma. In a report of a series of patients from Sweden, 42% of 111 patients with metastatic carcinoid tumors demonstrated biochemical responses, and 15% had objective tumor regression with interferon-α [26]. The same group reported a 47% biochemical response and 12% objective response in other neuroendocrine tumors [27]. In addition, there is literature to support biochemical improvement in patients with metastatic carcinoid tumor failing therapy

with octreotide when interferon-α is added to their therapy [28, 29]. Some authors have found the duration of benefit to be disappointingly short, and document excessive toxicity with high-dose regimens [30]. More recent investigations have focused on lower doses of interferon-α to attempt to minimize the myriad potential side effects of this drug, especially at high doses, such as fever, malaise, fatigue, bone marrow suppression, depression, and autoimmune phenomena. In addition, chemotherapy and interferon-α have been investigated, especially in combination with protracted venous infusion of 5-FU with some encouraging results in small studies [31]. The role of these combinations compared to the single agents themselves remains to be elucidated.

FUTURE DIRECTIONS

Clearly, more effective and less toxic therapy is needed for the treatment of metastatic neuroendocrine tumors. Ongoing clinical trials are investigating the use of yttrium-90-labeled somatostatin analogs in those patients with documented somatostatin receptors. We have investigated the pattern of molecular marker expression on a number of resected metastatic islet cell carcinomas and carcinoid tumors in an attempt to identify useful hypotheses for study of targeted therapy with novel agents. In a preliminary review of this analysis, c-*kit* and *Her-2/neu* were not felt to be attractive targets. Markers of increased angiogenesis were seen in some tumors, and there is an ongoing clinical trial of thalidomide (which has some antiangiogenic properties) in metastatic neuroendocrine tumors ongoing in this country. Expression of the epidermal growth factor receptor (EG ER) was noted, especially in gastrointestinal midgut carcinoid tumors (R. Lloyd, *personal communication*). We are currently developing a phase II study of a small molecule inhibitor of the EGFR tyrosine kinase for patients with metastatic neuroendocrine tumors. Advancing the care of patients with these diseases is difficult due to their heterogeneity and rarity. National and international cooperation is needed to go beyond the use of small single-institution phase II trials with differing criteria for response to evaluate new therapies.

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23 Radiation Treatment of Endocrine Tumors

SUNIL KRISHNAN, MD AND ROBERT L. FOOTE, MD

INTRODUCTION

Malignant tumors of the endocrine glands account for only 1.8% of the estimated 1.28 million new cases of nonskin cancer and 0.4% of the estimated 550,500 cancer deaths in the United States in 2002 [1]. Thyroid cancers account for the vast majority (approx 90%) of these cancers and about half of all the deaths due to endocrine cancers. Besides thyroid cancers, the most common neoplasms that are encountered in the clinical setting are tumors of the pituitary, parathyroid, adrenal gland, endocrine pancreas, and gonads. Multiple endocrine neoplasia (MEN) syndromes and carcinoid tumors are unique scenarios observed infrequently.

The evolving multidisciplinary approach to the treatment of all neoplasms is no different in the case of these endocrine tumors. A unique feature of many of these tumors, however, is their propensity to have tumor markers that can be used for diagnosis and followed prospectively to assess response to treatment. We will focus on the role of radiation therapy in the treatment of some of the more common endocrine tumors.

PITUITARY ADENOMA

Pituitary adenomas have an incidence of approx 1–14.7 per 100,000 people and an autopsy prevalence of approx 10–20%, with some autopsy series reporting a prevalence as high as 25% [2–4]. They comprise about 10–12% of all intracranial tumors. The peak incidence is between 45 and 50 yr of age with a female predominance [5].

Pituitary adenomas usually present with symptoms of mass effect or hormonal dysfunction (pituitary hypersecretion or hyposecretion). The most common presentation is that of an anterior pituitary tumor secreting excessive amounts of prolactin, growth hormone (GH), adrenocorticotropic hormone (ACTH), or thyroid stimulating hormone (TSH) resulting in amenorrhea–galactorrhea syndrome, acromegaly, Cushing's disease, or secondary hyperthyroidism, respectively. Neurologic sequelae arise from growth of a mass beyond the confines of the sella resulting in headache, visual loss (most frequently a superior temporal quadrantanopsia progressing to a bitemporal hemianopsia with diminished visual acuity), altered hypothalamic function (sleep, eating habits, and behavior) due to encroachment of the diaphragma sellae, optic chiasm/nerves, and hypothalamus, respectively. Further extension into the cavernous sinus may compress cranial nerves III, IV, V_1, V_2, or VI, causing specific neurologic symptoms. On rare occasions, obstruction of the third ventricle may cause obstructive hydrocephalus, cerebral extension may cause seizures, and sphenoid sinus extension may lead to cerebrospinal fluid (CSF) rhinorrhea and meningitis. Lastly, partial or total hypopituitarism may result from compression of the normal pituitary gland by a large macroadenoma.

When a patient's history and endocrinologic/neurologic exam suggests a diagnosis of pituitary adenoma, both an endocrinologic and an anatomic diagnosis are required to confirm this suspicion. Confirmation of endocrinologic diagnosis is achieved via measurement of basal and provoked hormonal levels, the usual basal hormones checked being GH, ACTH, cortisol, TSH, and luteinizing hormone (LH)/follicle stimulating hormone (FSH). Confirmation of anatomic diagnosis relies on high-resolution magnetic resonance imaging (MRI) with gadolinium enhancement with thin cuts through the sellar region to define the extent of supra- and parasellar extension and the positions of critical neurovascular structures (optic chiasm, carotid artery, and cavernous sinus). Visual field perimetry is performed in case of suprasellar extension or clinical evidence of visual deficit(s), and computed tomography (CT) scanning may help define bony erosion. In addition, it is worth considering the possibility of a MEN type 1 syndrome.

The most crucial components of management decision-making involve tumor size (which predicts tumor behavior, resectability, and treatment outcome) and hormonal activity (which defines the role of medical therapy and permits functional outcome assessment). Microadenomas (<10 mm in diameter) are more common than macroadenomas (>10 mm in diameter) and are often asymptomatic, despite typically being hormonally active. Macroadenomas are more likely to be hormonally inactive. When hormonally active, microadenomas are likely to be ACTH secreting while macroadenomas are more likely to be GH or prolactin secreting.

Once the diagnosis is established, the goal of treatment is to achieve restoration of endocrinologic and neurologic function while achieving local control of tumor. The options include pharmacologic inhibition of pituitary hormone secretion, microsurgical excision of the tumor, and conformal irradiation. Pharmacologic inhibitors include bromocriptine, a dopamine analogue

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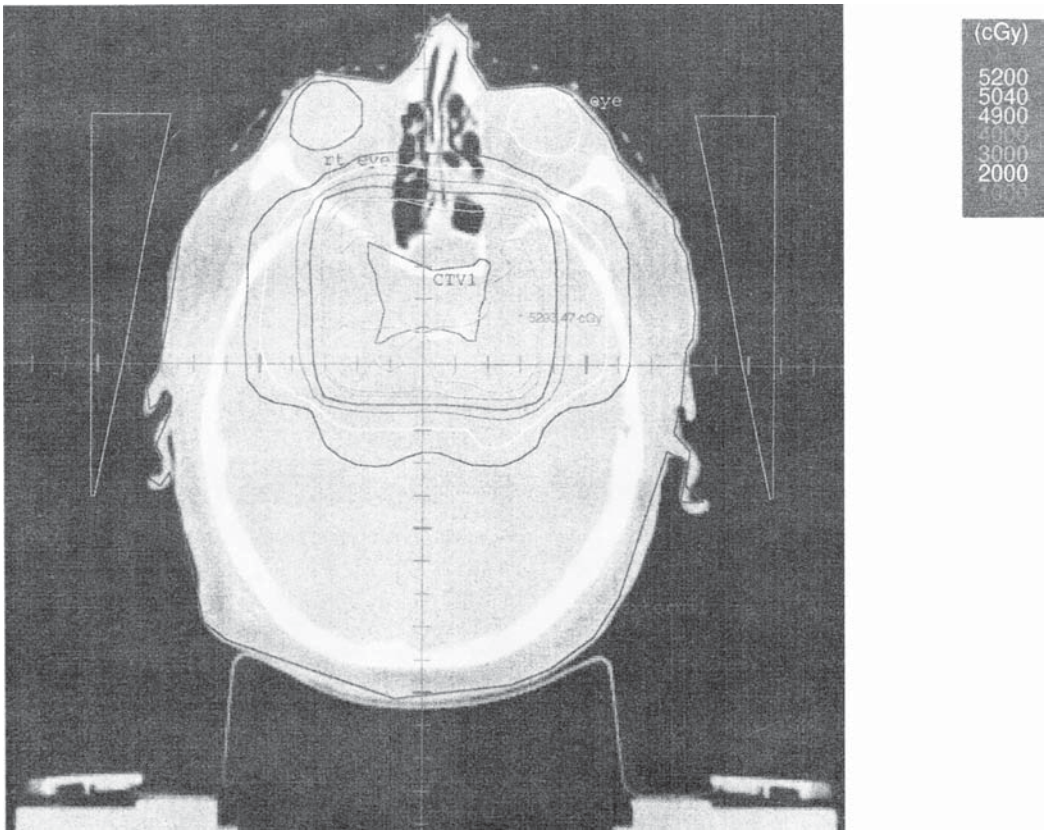

Figure 1 Axial CT scan image obtained for computerized treatment planning for a large nonfunctioning pituitary adenoma involving the right cavernous sinus. The CTV1 (clinical target volume 1) was obtained by fusion of the treatment planning CT scan images with MRI images obtained in the same patient position (mask immobilization). This volume received a dose of 50.4 Gy (*light blue line* fully encompassing the CTV1; the interrupted line is the 52 Gy isodose line) in 28 fractions of 1.8 Gy each. Dose falloff beyond the 30 Gy isodose line (*red line*) limits normal brain dose.

that serves to accentuate the physiologic inhibition of prolactin secretion; octreotide, a somatostatin analogue that reduces GH secretion; and metyrapone, an inhibitor of adrenal cortical cortisol production. Microsurgical excision usually employs a transseptal trans-sphenoidal approach to resect the entire tumor. Conformal irradiation may be administered either as fractionated external-beam radiation therapy (RT), hypofractionated stereotactic radiation therapy or radiosurgery (linear accelerator, Gamma Knife, or heavy ion beam based therapy).

A representative fractionated external-beam RT plan is outlined in Fig. 1. Multiple noncoplanar beams are used to conform the radiation dose three-dimensionally to a treatment volume encompassing the tumor (defined by fusion of the planning CT to an MRI) and a margin (to account for uncertainty of tumor extension, patient setup variability, and dose buildup within the field). Alternatives include moving arc fields and segmental rotational fields. Similar planning techniques using stereotactic immobilization frames are used for fractionated stereotactic radiotherapy.

In contrast, hypofractionated irradiation uses just a few treatment sessions (in the case of "radiosurgery," just a single session) to conform high-precision, large-dose radiation with steep dose gradients to small volumes so as to protect surrounding normal tissue. A representative Gamma Knife radiosurgery treatment plan is outlined in Fig. 2. Cobalt-60 sources (201 of them radially arranged in shielded collimator helmets) are used to aim narrow beams of radiation to stereotactically localized target volumes around isocenters placed within tumors. Similar treatment can be accomplished using a linear accelerator (linac) to rotate around isocenters placed within tumors or using heavy ion beams (protons, helium) that also have steep dose gradients (Bragg-peak effect).

Nonsecreting tumors may be treated surgically, with radiation alone, or with a combination of both. Given that most of these are macroadenomas at presentation and often have mass effects, surgical resection is often necessary yet incomplete, rendering recurrence rates ranging widely from 6% to 69% (median 23%) [6]. Primary RT also has a wide recurrence rate ranging from 7% to 50% (median 25%) [6]. Postoperative RT reduces the recurrence rate to 11%, conferring a 90% tumor control rate at 10 yr [6].

Prolactinomas are the most common secreting tumors. Microprolactinomas are usually treated with bromocriptine therapy alone and may not require surgery or RT. In about a third of all patients long-term medical therapy can be discontinued without prompting a relapse [7]. Macroprolactinomas with high pretreatment prolactin levels and mass effect or apoplexy are treated surgically and may be given bromocriptine preopera-

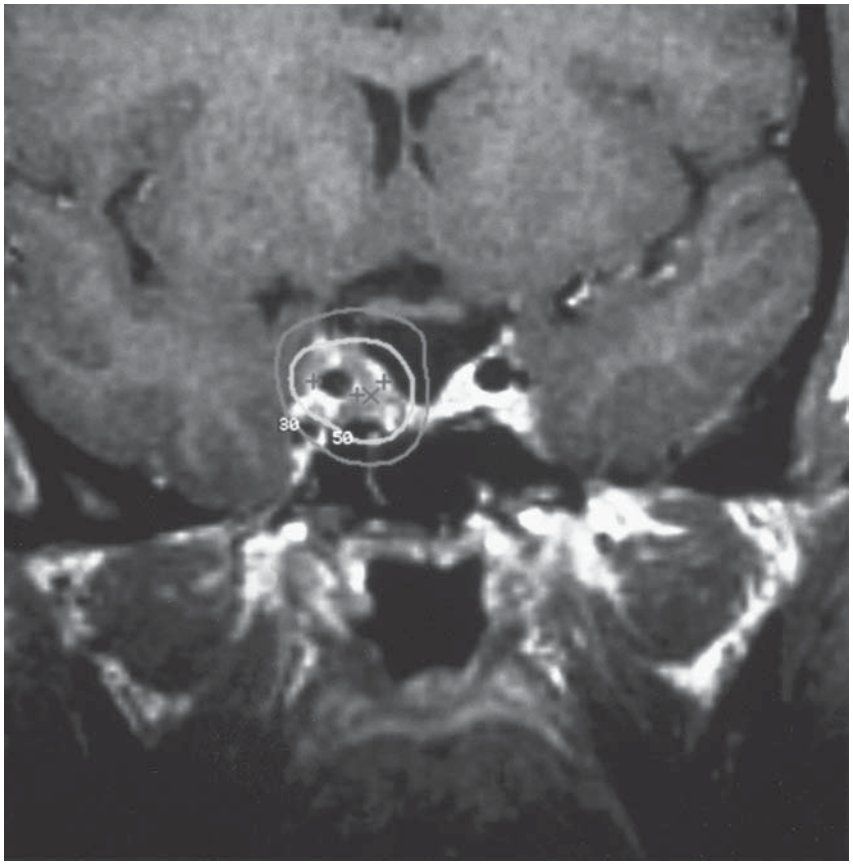

Figure 2 Coronal MRI obtained for computerized treatment planning for a recurrent pituitary adenoma involving the right cavernous sinus. A dose of 15 Gy was administered to the 50% isodose line (*yellow*). The maximum dose to the tumor was 30 Gy. The dose to the optic chiasm was <10 Gy (30% isodose line, *green*). (Color illustration appears in insert following p. 148.)

tively to decrease prolactin levels and tumor size. Despite this, incomplete surgical resection of macroprolactinomas results in high recurrence rates (averaging roughly 50%) [8]. Subtotally resected macroadenomas may be observed prospectively for tumor progression or treated postoperatively with RT. It is worth noting that persistently high prolactin levels may be due to pituitary stalk effects induced by tumor, radiation, or surgery. Primary RT is usually reserved for medically uncontrolled patients who are poor surgical candidates, and leads to a 5-yr prolactin normalization rate of roughly 50% [5]. Inoperable recurrences following surgery are often treated with external beam RT or stereotactic radiosurgery.

Acromegalic patients are also treated surgically wherever possible. Large tumor size, high pretreatment levels of GH, and the presence of coexistent prolactin-secreting elements confer a poorer prognosis. Normalization of somatomedin C levels and reduction of GH levels (<5 ng/mL) are routinely used as biochemical benchmarks of tumor control. Postoperative RT is generally reserved for instances when persistent hormone elevation is overtly symptomatic and refractory to medical therapy. Postoperative RT results in a nearly 80% tumor control rate [6]. Primary RT confers a 70–90% biochemical control rate [6]. It is worth noting that the GH level decreases at a rate of 10–30% per year. Dose–response data for these tumors suggest an increasing tumor response with doses up to 45–50 Gy, beyond which there does not seem to be any increased response [9–13]. Inoperable recurrences following surgery are often treated with external beam RT or stereotactic radiosurgery.

Cushing's disease usually arises from a microadenoma. If the microadenoma can be identified intraoperatively, a rapid cure can be accomplished surgically in up to 90% of patients. Postoperative RT in the setting of persistent or recurrent disease results in an 80% control rate (defined variably as clinical remission, biochemical remission, and lack of radiographic progression). Primary RT reduces hypercortisolism in 50–70% of patients. Medical therapy is seldom used.

Radiosurgery appears to be a possible treatment alternative for all pituitary adenomas in experienced centers, especially in patients with adenomas smaller than 25–30 mm with a minimum distance of 2–3 mm from the optic apparatus. Encouraging results have been reported with all forms of radiosurgery for primary treatment. Gamma Knife radiosurgery literature suggests a roughly 40–100% GH normalization at 2 yr [14–16] and 30–60% prolactin normalization at 2 yr [14,17]. These series report local control rates of >90%. Linac-based radiosurgery also results in >80% local control and comparable hormone normalization rates, albeit with greater neurologic toxicity (optic nerve damage and temporal lobe necrosis) when single isocen-

tric techniques are used [18,19]. Although lower complication rates were noted in one series [20], there has been a shift toward use of fractionated stereotactic radiotherapy or multiple isocentric techniques [19,21]. Reported hormone normalization rates at 5 yr are 50% for acromegaly and 80% for Cushing's disease with proton beam therapy [22]. Although these techniques have traditionally been reserved for retreatment of previously irradiated cases or instances where recurrence is noted at the cavernous sinus, there is insufficient durable control data to make them obvious choices as first-line radiation therapy [23].

Using modern equipment and techniques, the toxicity of radiation therapy is expected to be rare. Hypopituitarism is noted in 13–56% of patients and may be the result of a combination of factors including pretreatment tumor effect, surgery, and radiation [6]. With multiple non-coplanar beams the risks of radiation-induced brain necrosis is believed to be <1% [24]. Limiting the dose to the optic nerves and chiasm to 50 Gy reduces the risk of optic pathway dysfunction to <1%. For single-dose radiosurgery, a maximum dose of 10–12 Gy to a measurable partial volume of the optic pathway seems to be safe [25, 26]. Data derived from older techniques suggest a cumulative risk of developing a secondary brain tumor over the first 20 yr after RT of 1.9% and the relative risk (as compared to the normal population) of 9.4 [6]. Given that the median time to occurrence of second malignancies is about 10 yr, it is not surprising that reports of radiosurgery-induced second malignancies are sparse in the literature. Potential advantages of radiosurgery, as compared to conventional radiation therapy, include lower dose to surrounding normal tissues (brain, functioning pituitary, optic apparatus), shorter treatment time (1 d vs several weeks), and more rapid normalization of elevated hormone levels.

THYROID CANCERS

Thyroid cancers have an incidence of about 18,000 per year in the United States. Ninety five percent of these are differentiated (follicular or papillary) cancers. Differentiated cancers account for approx 1200 deaths annually in the United States. While there has been an increase in incidence of these cancers, there has also been a decrease in mortality (especially in women). Ten-year survival for papillary cancer is 93% and for follicular cancer it is 85%.

Thyroid cancers usually present as asymptomatic nodules localized in the neck but may occasionally present with nodal or distant metastases or with mass effects such as dysphagia, hoarseness, stridor, and dyspnea. Most patients are clinically euthyroid.

Confirmation of malignant histology is usually accomplished by a fine-needle aspirate (see Chapter 17 for details). The most common histologies are papillary, follicular, medullary, and anaplastic carcinomas and diffuse large cell or mucosal-associated lymphoid tissue (MALT) lymphomas (non-Hodgkin's type).

For differentiated thyroid carcinomas, the TNM staging system incorporates age, tumor size, nodal status, and presence of distant metastases to group tumors into different prognostic categories [27]. Other stratification strategies include the AGES (age, grade, extent, size) [28], AMES (age, metastases, extent, size) [29], and MACIS (metastases, age, completeness of resection, invasion, size) [30], all of which have been shown to predict prognosis. Knowing the prognosis, the aggressiveness of initial treatment can be tailored to the likelihood of recurrence and the potential success of initial salvage attempts. This is particularly relevant because roughly one third of all patients recur, two thirds of these being local-regional recurrences [31].

The initial treatment of choice is surgery, which usually involves a total or near-total thryoidectomy (including a lymph node dissection for papillary carcinomas that have a predilection for lymphatic spread) [32]. It is customary to obtain a whole body radioiodine scan (WBS) a few weeks after surgery to quantify residual thyroid tissue and distant metastases, thus helping to guide further treatment.

The thyroid remnant refers to the residual macroscopically normal thyroid tissue after thyroidectomy. In high-risk patients, the rationale for thyroid remnant ablation is to increase sensitivity of ^{131}I scanning by eliminating uptake by normal residual tissue, improve the sensitivity of thyroglobulin measurements as a marker for recurrence, and destroy occult cancer. This is believed to lower recurrence rates [31,33–35], lower pulmonary metastases rates [35,36], and decrease mortality in high-risk patients [34,35]. The treatment usually involves use of 30–100 mCi of ^{131}I depending on the size of the remnant. To enhance therapeutic efficacy, it is preferred that low dose (2–3 mCi) ^{131}I is used for the WBS and minimal iodinated contrast is used for staging CT scans. A 4–7-d post-ablation WBS may be performed to document efficacy of treatment. Subsequently, TSH is suppressed with thyroxine to just below the lower limit of normal levels in low-risk patients and more aggressively in higher risk patients or when there is residual untreated disease. Followup includes clinical examination aided by an ultrasound if necessary, thyroglobulin level measurement, TSH check, and WBS after thyroxine withdrawal or with recombinant human TSH administration.

Although efforts to improve on surgical outcome have focused mainly on radioiodine treatment in patients with a high risk of recurrence, there seems to be an evolving role for external-beam RT in their management. The rationale for inclusion of radiation therapy up front in the management of this subset of patients is to:

1. Treat areas that are known to take up less radioiodine (extrathyroidal or extranodal extension, which are known poor prognostic indicators [29,37–41]).
2. Treat carcinomas that fail to concentrate and retain radioiodine (20% of all differentiated thyroid cancers; patients with Hürthle cell tumors and patients above age 40 who commonly have less sodium iodide symporter [42–44]).
3. Spare some of the systemic toxicity of radioiodine treatment (when adequate treatment of residual disease entails excessive doses of radioiodine or when repeated treatments are likely to be necessary, increasing cumulative systemic toxicity [45]).
4. Spare some of the morbidity associated with uncontrolled local-regional cancer, such as obstruction of the esophagus and/or trachea, need for a laryngectomy, neurovascular compromise, pain, hemorrhage, and the need for repeated surgical procedures.

Nonrandomized studies have identified a potential role for adjuvant treatment of high-risk patients with external-beam RT [46,47], documenting improved local control with no survival

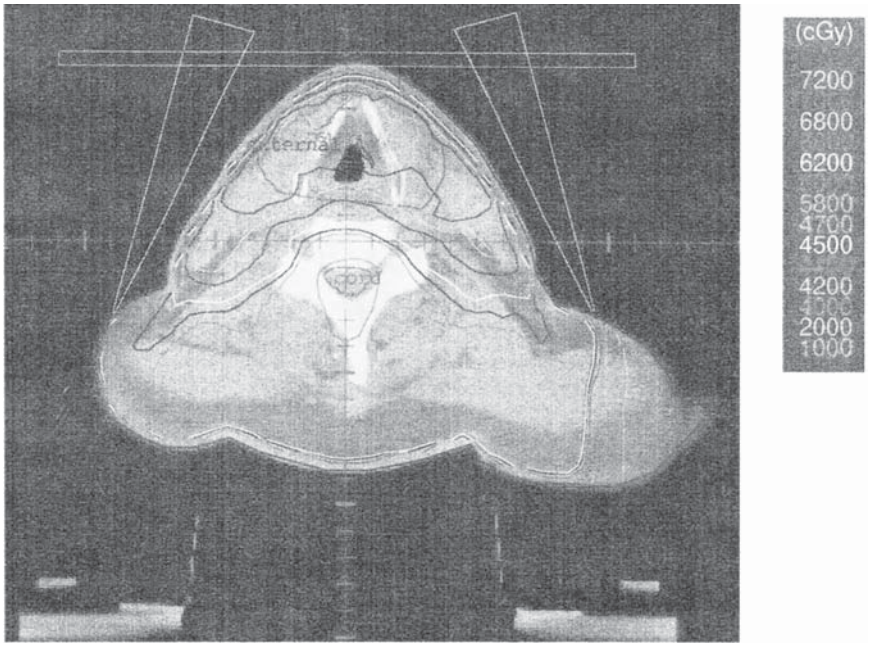

Figure 3 Axial CT scan image through the thyroid gland in a patient with thyroid bed recurrence after thyroidectomy for papillary thyroid cancer. A dose of 70 Gy was administered to the thyroid recurrence encompassed by the *innermost dark purple line*. A dose of 66 Gy was administered to the thyroid bed volume encompassed by the outer *dark purple line*. A dose of 60 Gy was administered to the regional lymph nodes encompassed by the *outer brown line*. The dose to the spinal cord was <47 Gy (*green line around the cord*).

benefit [48–54]. In one study, there was a cause-specific survival benefit noted among papillary cancers with microscopic residual cancer [33]. The inclusion criteria for receiving external-beam RT have included advanced age (>40 yr), extrathyroidal extension, high European Organisation for Research and Treatment of Cancer (EORTC) score, or other scoring systems for prognostic variables. Local recurrence has purportedly declined from 20–25% to 4–7% with the addition of external-beam radiation therapy in these studies, translating to a 65–85% decrease in local recurrence. This large a decrease in local-regional recurrence is likely to translate to a potential decrease in metastasis rate and improved survival, with enough patients treated.

In addition to the adjuvant treatment mentioned above, external-beam RT is used in the treatment of advanced inoperable primary or recurrent local-regional disease [33,48,49,55], radioiodine-resistant progressive disease [56] and bone or brain metastases. Treatment of the thyroid gland and its adjacent lymph node areas has historically been a challenging undertaking, inspiring a number of novel strategies to achieve adequate tumor doses while minimizing normal tissue doses. Anterior–posterior fields with lead blocks for the larynx and lungs, lateral fields, oblique fields, and centrally shielded arcs with one or two centers were the most common techniques employed in the past. More recent treatment modalities include shaped electron fields, three-dimensional conformal multiple-beam treatments, and intensity-modulated RT (IMRT) treatments. The last of these is a method in which normal tissue anatomy and dose limits are utilized by a computer to select an ideal treatment plan that delivers the required tumor dose using small "beamlets" of varying intensity, instead of manually selecting potential treatment fields of the same beam intensity and hoping to limit normal tissue doses [57]. A representative three-dimensional conformal treatment plan is shown in Fig. 3. Typical doses are 50–60 Gy over 5–6 wk for microscopic disease (adjuvant treatment). Doses in the range of 60–70 Gy are employed for gross residual disease, with roughly 60% relapse-free survival at 5 yr and 40% at 15 yr. Treatment volumes typically encompass all lymph node regions "at risk" (typically from the mandible to the carina). There may be a role for the use of amifostine as a radioprotector to limit the degree of radiation-induced xerostomia by protecting the salivary glands [58].

We have a limited experience using radiation therapy in the management of Hürthle cell carcinoma of the thyroid gland. We have found it to be a radiosensitive malignancy. We have treated five patients with adjuvant radiation therapy. Indications included large tumors (>6 cm), extensively invasive tumors (trachea, muscle, and vascular structures), lymph node metastases, and positive surgical margins. Four patients remained free of local or regional tumor recurrence 12.4–47.7 mo following radiation therapy. We have treated five patients with unresectable recurrences within the neck. Three patients enjoyed local and regional tumor control for the remainder of their lives (18.9, 102.6, and 106.0 mo). We have treated 14 patients with 95 palliative courses of radiation therapy for symptomatic metastases. All patients experienced relief of pain and other symptoms for a median of 12 mo (range, 0.5–68 mo). Overall, 33% of the sites treated required retreatment. If a dose of about 25 Gy is utilized, only 16% of metastatic sites will require retreatment and the median duration of palliation is extended to 14 mo.

In addition to treatment of differentiated thyroid cancers, external-beam RT has been used in the nonsurgical treatment of medullary thyroid cancer. Medullary thyroid cancer, a tumor

of the parafollicular C cells that produce calcitonin, accounts for approx 10% of all thyroid malignancies. An estimated 75% of these are sporadic, while the remaining 25% are familial which tend to be multifocal bilateral tumors. The hereditary medullary thyroid cancer syndromes include MEN 2A, MEN 2 B, and familial medullary thyroid cancer without other malignancies. MEN 2A patients tend to have accompanying pheochromocytomas and parathyroid hyperplasia/ adenomas. MEN 2B patients develop medullary thyroid cancers in association with pheochromocytomas, mucosal neuromas, intestinal ganglioneuromas, and Marfanoid habitus. Once the diagnosis of a thyroid mass has been established as medullary thyroid cancer with a fine-needle aspirate, it is customary to obtain preoperative measurements of serum calcitonin and carcinoembryonic antigen (CEA) levels, screen for pheochromocytomas with urinary catecholamine measurements, and screen for MEN 2 syndromes by genetic testing for *RET* protooncogene mutations. Primary treatment involves total thyroidectomy with parathyroid autografting, central lymphadenectomy, and in larger tumors with nodal involvement, an ipsilateral functional neck dissection. Persistent or recurrent disease may be treated with further surgical debulking. While there are no prospective randomized trials evaluating the role of external-beam RT in the treatment of medullary thyroid cancer, there are some retrospective reviews that shed light on this subject. In locally advanced disease, external-beam RT was shown to decrease local relapse rate from 59% to 29% without impacting on overall survival in one series [59]. In another series of high-risk patients with microscopic residual disease, extraglandular extension or lymph node involvement, a median dose of 40 Gy in 20 fractions was noted to increase the local-regional relapse-free survival rate from 52% to 86% [60]. However, 30% local recurrence rates within an irradiated field after 54 Gy [61] and even decreased survival among patients treated with radiation therapy [62] have been reported. In general, gross residual unresectable disease and recurrent locoregional disease seem to derive a local-regional control benefit from external-beam RT [63,64]. While adjuvant treatment of microscopic residual disease with radiation therapy is not routine, it would seem to be a reasonable option for patients at high risk of local-regional relapse [60]. Radioimmunotherapy with ^{131}I -labeled CEA monoclonal antibody protocols are an option in case of inoperable widely metastatic medullary thyroid cancer and somatostatin analogs are used for symptomatic disease [63].

Anaplastic thyroid cancer has a universally dismal prognosis with survival beyond 1 yr being very uncommon. The hallmark of the disease is a rapidly enlarging mass with pain and pressure effects. Although no optimal treatment is available, a multimodality approach consisting of surgery, radiation, and chemotherapy seems to be the preferred approach. While most series report long-term survivors only among surgically resected patients [65–70], the high morbidity of this procedure in the face of a poor prognosis may warrant its judicious use [71]. Radiation therapy in conjunction with doxorubicin chemotherapy results in improved local-regional control and survival in some series [64,70,72] although not in others [73]. Hyperfractionated radiation therapy (more than one treatment per day) has been employed in some series as a means of adequately treating a tumor with a high proliferation rate, while limiting normal tissue toxicity [40,65,66,74]. Despite the promise of improved outcomes with aggressive multimodality treatment regimens, the outlook for anaplastic thyroid cancer patients is still very grim.

Thyroid lymphomas are a distinct subset of non-Hodgkin's lymphomas, characterized by localized disease with late relapses. They are most commonly diagnosed by fine needle aspiration to be MALT lymphomas or diffuse large cell lymphomas of intermediate or high-grade morphology. Although most of these tend to be limited to the thyroid (Ann Arbor stage IE), a complete workup to look for other sites of involvement is usually recommended. Often there is an underlying history of Hashimoto's autoimmune thyroiditis. As with other lymphomas, the treatment is nonsurgical.

The management of stage I/II diffuse large cell lymphoma is combined modality therapy with radiation and CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) chemotherapy based on the results of randomized studies by the ECOG (Eastern Cooperative Oncology Group) and SWOG (Southwestern Oncology Group). The ECOG group compared eight cycles of CHOP chemotherapy to the same chemotherapy with 30–40 Gy radiotherapy to a localized area including the thyroid and regional nodes. Combined modality therapy improved disease-free survival and time to progression without improving survival [75]. The SWOG study compared eight cycles of CHOP to three cycles of CHOP followed by 40–55 Gy localized radiotherapy [76]. Although there was an improved failure-free survival and overall survival rate noted at 4 yr for combined modality therapy, this advantage was lost with further follow-up extending to 10 yr. However, combined modality therapy is still recognized as the best standard treatment for stage I/II diffuse large cell lymphoma patients based on the survival advantage through the first 9 yr and the lower toxicity noted in this study [77]. The standard treatment for bulky stage II or stage III/IV diffuse large cell lymphoma is eight cycles of CHOP chemotherapy [78]. CHOP chemotherapy plus rituximab (a chimeric anti-CD20 IgG_1 monoclonal antibody) has been shown to improve event-free survival and overall survival in elderly patients [79].

The treatment options for stage I/ II low-grade MALT lymphomas include localized low-dose radiation therapy (25–35 Gy), oral chlorambucil, or intravenous chemotherapy (cyclophosphamide, vincristine, and prednisone) [80,81]. Given the ease of administering low-dose radiation therapy, this is often the best choice of treatment. In patients with stage III/IV disease, treatment with CHOP chemotherapy in SWOG protocols did not lead to better failure-free and overall survival rates than that noted for follicular lymphomas, indicating an aggressive course of disease [82].

PARATHYROID CANCERS

Carcinoma of the parathyroid gland is a rare cause of hyperparathyroidism, accounting for fewer than 1% of patients with hyperparathyroidism. Patients with parathyroid carcinoma usually present with profound symptoms of hyperparathyroidism with highly elevated serum parathyroid hormone (PTH) levels, hypercalcemia, and hypophosphatemia.

The single most effective therapy for localized parathyroid carcinoma is complete en bloc resection of the primary lesion with ipsilateral thyroid lobectomy and cervical lymph node dissection at the time of the initial operation. Owing to the rarity of the disease, the technically challenging nature of the surgical procedure, and occasional late intraoperative recognition of disease, many patients fail to receive such treatment. The resultant subsequent tumor progression, often despite such surgery, is estimated at about 50% without adjuvant treatment [83]. Reoperation is recommended in patients with local-regional persistent or recurrent disease with no distant disease because it relieves symptoms of hypercalcemia, and it temporarily normalizes serum calcium and PTH levels in most patients. For patients who have unresectable parathyroid carcinoma, a protocol-based treatment with chemotherapy and external radiotherapy should be considered [83]. Many studies addressing the role of radiation therapy in the management of parathyroid cancer have reported minimal success at reducing hormone production and tumor growth [84–90]. However, two studies and our own experience suggest that adjuvant radiation therapy to the tumor bed might decrease the strong predilection for local progression and death due to hypercalcemia [91,92]. Indications for postoperative radiation therapy include recurrent cancer; close or positive margins; tumor invading trachea, esophagus, or neurovascular structures; and lymph node metastases. A dose of 50 Gy is usually administered to the operative bed and regional lymph nodes with an additional boost dose of 10 Gy to the operative bed. A total dose of up to 70 Gy is given for microscopic or gross residual cancer. Acute reactions include dermatitis, laryngitis, and esophagitis. Late sequelae are uncommon but may include primary hypothyroidism, laryngeal edema, cartilage necrosis, esophageal stenosis, myelitis, and pulmonary fibrosis.

PANCREATIC ISLET CELL CANCERS

Islet cell tumors of the pancreas are rare, indolent, neuroendocrine tumors. Approximately 50% of the patients diagnosed with these tumors present with symptoms related to various biologically active hormones that are secreted by these neoplasms. Currently, the only curative treatment for islet cell tumors is complete surgical resection. Unresectable, locally advanced cases are usually treated with the somatostatin analog octreotide, and chemotherapy (interferon-α, streptozotocin, 5-fluorouracil) may help control hormone secretion and stabilize tumor growth. There is a scarcity of literature on the use of external radiation therapy in this setting, with some case reports documenting good outcomes [93,94]. As a standard practice, radiation therapy is not considered an integral part of treatment of pancreatic islet cell tumors.

CARCINOID TUMORS

Carcinoid tumors are rare, indolent neuroendocrine cell tumors that most commonly involve the lungs, bronchi, and gastrointestinal tract. They are traditionally classified as fore-gut carcinoid tumors (originating in the lungs, bronchi, thymus, stomach, or duodenum), midgut carcinoid tumors (small intestine, appendix, and proximal colon), and hindgut carcinoid tumors (distal colon and rectum). The characteristic membrane-bound neurosecretory granules seen in neuroendocrine cells typically contain serotonin and other vasoactive substances. In addition to serving as histologic identifiers, these substances serve as tumor markers (serum chromogranin and urine 5-hydroxyindoleacetic acid being the classical examples). Furthermore, somatostatin receptor scintigraphy serves as a very sensitive localization procedure.

Resectable disease is usually treated surgically when possible. In patients with unresectable symptomatic disease, a number of treatment options are available including chemotherapy, arterial embolization, somatostatin analog treatment, and *in situ* radiation therapy. *In situ* radiation therapy capitalizes on the expression of the somatostatin receptor type 2 on >80% of all carcinoid tumors, allowing for its targeting with its ligand bound to a radioisotope-emitting short-range decay particle to tumor cell nuclei. ^{111}In-pentetreotide and a ^{90}Y-DOTA–lanreotide derivative are two such radiolabeled somatostatin analogs shown to have significant early response rates (objective and clinical symptom benefit) with minimal toxicity [95–97]. ^{131}I-meta-iodobenzylguanine (MIBG) (used routinely for diagnostic imaging purposes) has also been used as a means of administering therapeutic-range doses of localized radiation to tumors that exhibit high uptake rates [98–100]. These results would make a compelling case for the efficacy of external-beam RT for unresectable symptomatic disease. However, there are few reports in the literature attesting to the efficacy of external-beam RT in the management of patients with carcinoid tumors [101–104]. External irradiation has generally been reserved for amelioration of symptoms caused by bone, brain, and skin metastases.

CONCLUSION

Radiation therapy can be beneficial in the management of a variety of endocrine tumors. Typical indications include large or small, unresectable or recurrent benign or malignant tumors of the pituitary, thyroid, or parathyroid glands. Typical doses utilized for benign tumors are 45–50 Gy given over a 5–6-wk period of time. Malignant tumors require 60–70 Gy administered over a 6–7-wk period of time. Radiation therapy may also be beneficial in preventing recurrent tumor with the associated morbidity and mortality in patients with completely resected yet advanced (invasive, nodal metastases, close or positive margins) thyroid or parathyroid cancer. Radiation therapy can palliate distressing symptoms due to bone or soft tissue metastases for almost all malignant tumors. A typical dose would be 30 Gy given over 2 wk. The radiation oncologist can provide valuable insight in the comprehensive evaluation and to the multispecialty team caring for the patient with advanced or metastatic endocrine neoplasia.

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24 Future Directions in Endocrine Pathology

RICARDO V. LLOYD, MD

INTRODUCTION

Many rapid advances are being made in understanding the pathogenesis of endocrine tumors and in developing methods to treat tumors and other endocrine disorders more effectively. Some of these new technological developments including gene expression profiling, proteomics, and high-throughput tissue microarrays as well as functional gene analyses with small interference RNAs have led to the discovery of new genes and provided information about the functions of many genes. Advances in tumor localization, developments in pharmacology, and gene therapy approaches should lead to new and effective methods of treating endocrine disorders.

PATHOGENESIS OF ENDOCRINE TUMORS

As some of the preceding chapters in this book have clearly shown, the pathogenesis of endocrine tumors is extremely complex, and the mechanisms of tumor development remain quite elusive. To make the most rapid progress in elucidating the pathogenesis of endocrine tumors, investigators will have to concentrate on specific organs, as the mechanisms of genetic instability leading to tumorigenesis are quite different in various endocrine tissues. The available evidence would suggest that specific oncogenes and tumor suppressor genes are altered in different tumors even within the same organ. For example, in the anterior pituitary gland, the heterogeneity of genetic instability leading to tumor development may extend to specific cell types. The available evidence suggests that there are cell type specific genetic alterations leading to tumorigenesis in different cell types in the anterior pituitary (refs. [1–6] and Chapter 5). In the case of tumor suppressor genes such as the multiple endocrine neoplasia (MEN) type I and type 2 genes that affect various endocrine tissues including the pituitary, parathyroid, pancreatic islets, and adrenal medulla, the findings resulting from genetic alterations in these MEN genes have been quite variable with some target tissues affected more commonly than others [7,8]. For example, in the parathyroid gland, inactivating mutations in tumors with loss of heterozygosity at 11q13 has supported a role of the *MEN1* tumor suppressor gene in a subset of sporadic parathyroid tumors [9]. Similar studies in gastrinomas have shown that mutations in the *MEN1* gene are important in a proportion of sporadic gastrinomas [10]. In contrast, tumors of the pituitary gland in patients with MEN1 are associated with alterations in the *MEN1* gene whereas sporadic pituitary tumors rarely have alterations in the *MEN1* gene [11–13].

The MEN 2A, 2B, and familial medullary thyroid carcinoma gene which is the *RET* protooncogene, has been associated mainly with familial diseases affecting the thyroid C cells and adrenal medulla and to a lesser extent with sporadic diseases affecting these tissues [14–16]. The complexity in the expression of genes involving multiple endocrine familial syndromes highlights the paucity of information currently available to understand tumor pathogenesis. Newer approaches summarized below should lead to new aspects of gene discovery and accelerate knowledge about the etiology and treatment of endocrine tumors.

DNA MICROARRAY TECHNOLOGY Recent advances in DNA array technology have allowed systematic approaches to biological discoveries that will have a major impact in biology, pharmacology, and medicine [17–24]. RNA expression profiles should provide a very precise and reproducible signature about the state of normal and neoplastic-specific cells and tissues that may reflect the functional state of the cells. This has many implications for tumor classification as well as for understanding pathogenesis of endocrine and other tumors. RNA expression profile arrays have been studied in various endocrine tissues including the pituitary [25–27], pancreatic islets [28], adrenals [29], and thyroid [30]. Studies in the pituitary have uncovered genes that are uniquely expressed by different types of pituitary tumors, which reinforces earlier observations that various tumor types within the same endocrine tissue may have different genetic alterations. Nonfunctional adenomas have been found to overexpress the folate receptor while growth hormone tumors overexpress ornithine decarboxylase [25]. Array studies in normal pancreatic islets have shown overexpression of transforming growth factor-β (TGF-β), thioredoxin-interacting proteins, and islet amyloid polypeptide. These studies highlight the importance of the TGF-β family in the regulation of islet cell function [28]. Recent DNA array studies with papillary thyroid carcinomas [30] have revealed that genes with increased expression included those encoding adhesion and extracellular matrix protein, while tumor suppressor thyroid function related proteins and fatty acid producing proteins were underexpressed [30].

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PROTEOMIC ANALYSIS Proteomic analysis is a logical extension of genomic analysis, because these approaches are an important component of functional genomics and will provide new information about the functions of specific genes. Determination of protein profiles using two-dimensional polyacrylamide gel electrophoresis and mass spectroscopy are the principal tools for analytical protein identification [30–36]. A recent study using proteomic analysis of thyroid tissue has shown upregulation of cathepsin B in thyroid neoplasms [31]. The field of proteomics holds great promise in providing new insights into the discovery and analysis of new proteins in endocrine cells and tumors.

LASER CAPTURE MICRODISSECTION AND HIGH-THROUGHPUT TISSUE MICROARRAY Because of the heterogeneity of endocrine and other tissue types, various approaches including laser capture and laser-assisted microdissection (LCM) have been utilized for more sophisticated tissue analyses such as cDNA array and proteomic analyses [35–37]. The application of LCM has led to a higher level of specificity in the downstream application of sophisticated technology to understand cell and tissue functions. The use of high-throughput tissue microarray analysis to evaluate genes uncovered by cDNA microarrays and for other types of analyses has also accelerated the pace of gene discovery [38, 39]. With the use of many hundred specimens of a particular sample type, problems associated with tissue heterogeneity, which is common in endocrine tissues, can be rapidly evaluated in the detailed molecular profiling of tumors.

RNA INTERFERENCE RNA interference is rapidly becoming a powerful new tool for manipulating gene expression in mammalian cells. This new technology has potential utility in analyzing endocrine and other gene functions, high-throughput function-based genetic screens, and for development of new therapeutic tools [40–43]. This technique, which was first described in *C. elegans* and in plants, operates by using double-stranded RNA to silence gene expression at a posttranscriptional level. Exposure to double-stranded RNAs results in loss of mRNA. New mechanisms of silencing include RNA-induced silencing complex (RSC) which converts the silencing trigger to approx 21–25 nucleotide RNAs, and these small interfering RNAs (siRNAs) operate by joining an effector complex RSC that guides the complex to homologous substrates. This powerful new approach is being used to study all of the approx 19,000 genes in *C. elegans*, and all of the genes in the human genome will probably be examined by siRNA in the near future [40]. A great deal of knowledge about the function of many genes in endocrine tumors will soon be forthcoming with this new technology.

TUMOR LOCALIZATION AND DETECTION Many traditional and new techniques have been used to localize endocrine tumors including in vivo imaging with magnetic resonance imaging (MRI), positron emission tomography (PET), and computed tomography (CT). These radiologic techniques have provided a great deal of morphologic details in localizing endocrine tumors [44–46]. The use of octreotide to image growth hormone producing pituitary tumors [46] and sestamibi scans to localize parathyroid tumors [47] are examples of recent developments that have increased radiologic diagnostic accuracy. Newer techniques such as double-contrast-enhanced CT with effervescent granules along with glucagon narrow collimation have been used recently to detect small gastric carcinoids and some otherwise hard to detect neuroendocrine tumors [48]. We can anticipate that these techniques will continue to improve and allow us to detect smaller endocrine lesions in the future.

PHARMACEUTICAL THERAPIES

The rapidly emerging new technologies outlined in the preceding including cDNA arrays and RNA interference should accelerate the development of new pharmaceutical agents that will be effective in the treatment of endocrine tumors and other endocrine diseases. The use and analysis of traditional drugs have shown that these are more complex than previously realized. Although octreotide is useful for some pituitary, pancrea-a-tic, and endocrine tumors, and gastrointestinal carcinomas, recent studies have elucidated the five types of somatostatin (SST) genes and receptors expressed by different tumors. In many endocrine tumors, SST-R2 and SST-R5 are the most commonly expressed receptor types [49–53]. The manufacture of subtype specific receptor antagonists may possibly enhance the efficacy of drugs such as octreotide, which are used to treat these tumors.

Other receptor-targeted therapies such as the epidermal growth factor receptor (EGFR) with drugs such as ZD-1839 (Iressa), which inhibits EGFR activation and affects downstream receptor-dependent processes in vivo, may assist in treating some endocrine tumors [54–56].

GENE THERAPY

The field of gene therapy as a method of treating neoplasms and other disorders has been making slow progress over the past few decades. Gene therapy approaches have been applied in preliminary experiments to endocrine tumors such as pituitary and thyroid tumors [57–60]. A common strategy in gene therapy is to use adenoviral vectors with specific promotors to express selectively marker genes or toxic genes in tissues of interest. The herpes simplex virus thymidine kinase gene has been used to induce cytotoxicity in pituitary adenoma cells following the administration of ganciclovir [57]. In other studies, the POMC promotor has been used in gene therapy strategies for treatment of pituitary tumors causing adrenocorticotropic hormone (ACTH)-dependent Cushing's syndrome [57]. Other investigators have used adenoviral β-galactosidase expression driven by human cytomegalovirus promotor or the human prolactin gene promotor with stereotaxic delivery in the ovine pituitary to produce cell-type-specific expression of an adenoviral transgene in mixed populations of the intact pituitary gland [58].

Anaplastic thyroid carcinomas are lethal endocrine cancers without effective means of treatment. Investigators have recently used adenovirus-mediated wild-type *p53* gene therapy with a replication-deficient recombinant adenovirus vector, which has led to a dose-dependent killing of normal and carcinoma cells, with more effective killing of the cancer cells [60]. As new methods of gene delivery are developed, gene therapy should become an important tool in treating highly lethal endocrine cancers such as anaplastic thyroid and adrenal cortical cancers.

STEM CELLS Stem cells are potentially very useful for treating many diseases including endocrine disorders, because of

their ability to undergo self-renewal and differentiation. The inability of many endocrine tissues to regenerate is well known, so embryonic stem cells (ES) as well as possibly adult stem cells may be useful in treating endocrine disease such as juvenile onset or type 1 diabetes mellitus [61–64]. Because human ES cells can proliferate indefinitely and differentiate into multiple tissue types, these cells could potentially provide an unlimited supply of tissues for human transplantation including islet cell transplantation. The recent studies with mesenchymal stem cells derived from adult bone marrow [62] and neural stem cells derived from adult mouse brain [63] are promising areas of exploration, especially given the close relationship between the neural and endocrine systems.

In summary, the many recent advances elucidating the pathogenesis of endocrine tumors and the development of new technological advances such as high-throughout RNA expression profiling, proteomics, and RNA interference should rapidly increase our knowledge of endocrine tumor pathogenesis. New diagnostic and treatment modalities including new pharmacologic agents and gene therapy approaches are emerging from these exciting advances for the treatment of a wide spectrum of endocrine disorders.

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Endocrine Pathology

Differential Diagnosis and Molecular Advances

Edited by

Ricardo V. Lloyd, MD, PhD

Department of Pathology, Mayo Clinic, Rochester, MN

The significant insights and new diagnostic possibilities arising from recent discoveries in cellular and molecular biology are transforming the field of endocrine pathology. In *Endocrine Pathology: Differential Diagnosis and Molecular Advances,* a panel of leading experts capture these dynamic changes by critically reviewing those molecular discoveries that can clarify the pathophysiology of endocrine disease processes and enhance the diagnosis of specific endocrine pathologies. Here, pathologists, endocrinologists, surgeons, medical oncologists, and radiation therapists discuss the treatment of endocrine disorders, especially tumors, with emphasis on in-depth differential diagnosis and on broadening the endocrine pathologist's ability to make accurate tissue diagnoses.

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