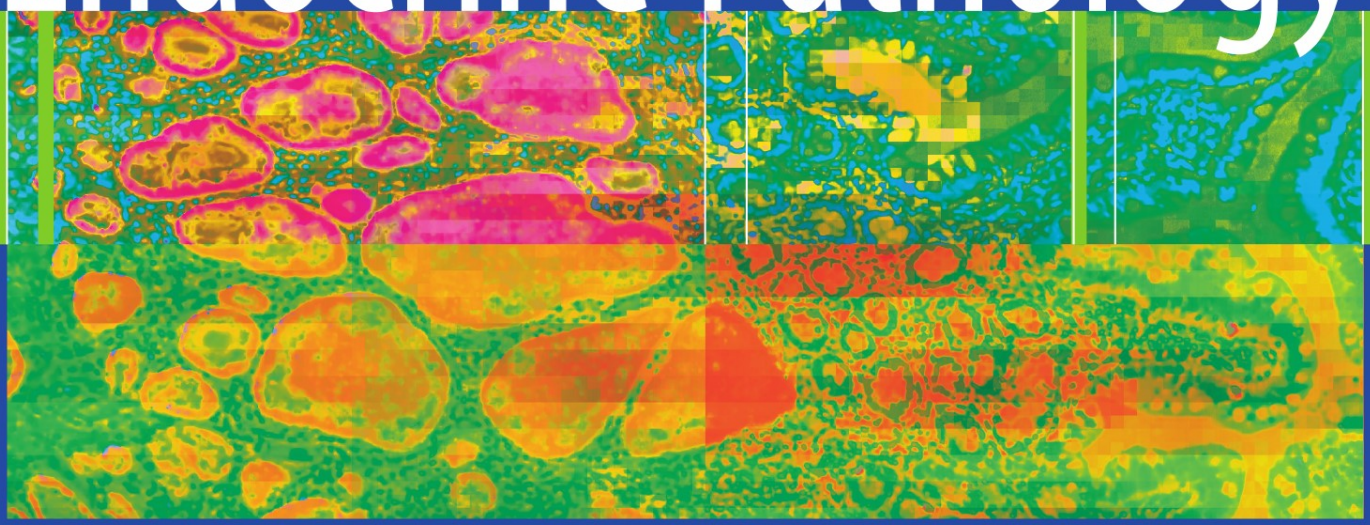
Ricardo V. Lloyd *Editor*

Endocrine Pathology



Differential Diagnosis and Molecular Advances

Second Edition

Springer

Endocrine Pathology

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Editor

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Preface to Second Edition

The second edition of this book attempts to capture the rapid developments in molecular advances in endocrine pathology that have occurred in the past few years.

This second edition continues the practice of utilizing the same general approach used by diagnostic pathologists as they examine endocrine lesions. Differential diagnoses based on the gross and histopathologic aspects of the lesions continues to be emphasized.

The field of endocrine pathology remains in rapid transition. The chapters in this book attempt to capture these dynamic changes by discussing the basic approach to diagnosing endocrine lesions in addition to examining the most significant developments in cell and molecular biology that help to understand the pathophysiology of disease processes. Many of these new observations will soon make their way to the diagnostic bench of the pathologist who now utilizes many immunohistochemical stains and a few molecular tools in establishing specific diagnoses of endocrine lesions. These new approaches are especially helpful when the pathologist receives a small biopsy with a few millimeters of tissues from a fine needle aspiration biopsy procedure.

The first chapter of this new edition is markedly expanded in attempts to capture most of the basic technical advances that have occurred in the past 5 years. An appreciation of the technical basis of these procedures provides insights for the critical evaluation of data generated with these techniques that the reader will encounter in the subsequent chapters.

New chapters providing more in depth examination of the endocrine thymus, endocrine lung, skin and placenta, as well as molecular developments in the analysis of pheochromocytomas and paragangliomas have been added. The importance of the medical work up of patients with endocrine disorders is emphasized in a new chapter on Biochemical Testing for Neuroendocrine Tumors. This new edition also contains many more color illustrations than the previous one.

The chapters on the treatment of endocrine disorders from the perspective of the surgeon, medical oncologists and radiation therapists who have major roles in the treatment of patients with endocrine disorders provides the pathologist with further insights into the role of each member of the endocrine working group in patient care.

We expect that the new technical and diagnostic advances that occurred between the first and second editions of this book will continue at the same rapid pace. It is only by learning about these advances as they emerge can we expect to deliver the best care to patients with endocrine disorders.

The editor wishes to thank the contributing authors who are outstanding endocrine pathologists, molecular pathologists or clinicians for their excellent contributions. In addition, we want to thank Richard Hruska, Humana Press and Springer Sciences and Business Media for making this second edition possible.

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Chapter 1
Methods in Cellular and Molecular Pathology

Paul Komminoth, Axel Walch, Martin Werner, and Aurel A. Perren

1.1 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, which have been applied in diagnostic and investigative endocrine pathology. General principles and possible applications of each method will be 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 which are cited in the text.

1.2 Immunohistochemistry

Immunohistochemical techniques, which have been 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 great variety of antigens, not only in fresh but also formalin-fixed and paraffin-embedded tissues or cells.

1.2.1 Principles

Immunohistochemistry is a method, which 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 five to ten amino acids. The technique of immunohistochemistry is, in principle, composed of two steps. In the 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 the second step, the binding between antigen and antibody is visualized using direct or indirect detection techniques.

1.2.2 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 splenic 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 above-mentioned systems, there also exist cell-free methods for the production of monoclonal antibodies.

Recently, chimeric (hybrid) antibodies have been developed combining the advantages of monoclonal with the specificity of human antibodies [4–7].

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1.2.3 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 pathology. Fixation leads to denaturation or precipitation of protein resulting in a masking of antigen structures (epitopes). Thus, it is frequently necessary to pre-treat 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 [8–10]. Epitope retrieval should merely cleave the fixation-caused bonds, resulting in a reconstruction of the original three-dimensional structure of the epitope. Depending on the cleavage sites of the proteolytic enzymes used, the antigenic epitopes themselves may be altered. 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, which 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 alliance. It plays only a minor role which heating source is used to provide the energy to break crosslinks [11]. Either 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 ethylene-diamine-tetra acetic acid (EDTA) buffers [11]. 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 [12] 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 [11]). For some rare antibodies, a combination of protease pretreatment and heat-induced antigen retrieval has to be done 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.

1.2.4 Methods of Immunohistochemistry

Depending on the detection system, dewaxed, rehydrated and pretreated slides or cells are pre-incubated with buffers containing blocking reagents for endogenous peroxidase and non-specific antibody binding sites. Next, the primary either polyclonal or monoclonal antibody appropriately diluted in buffer (usually PBS) is applied and incubated from 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. 1.1). 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

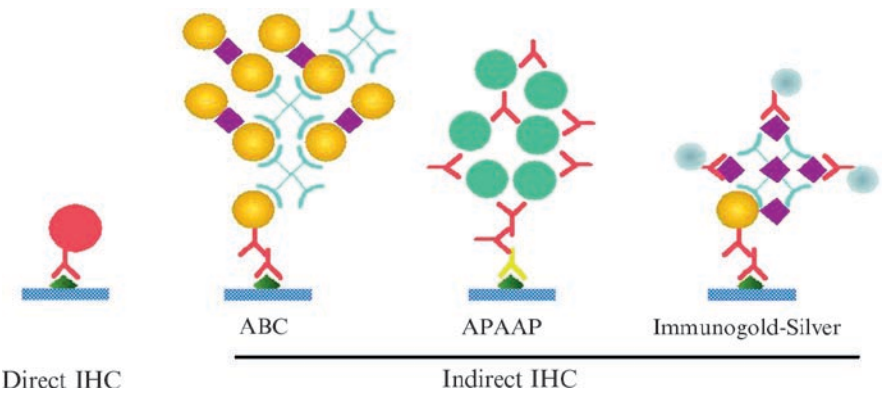

Fig. 1.1 Schematic representation of direct and indirect immunohistochemistry

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 (1) the enzyme *horseradish-peroxidase* (HRP), which can be conjugated to antibodies and other reagents, is the most widely used immunolabeling system. Using H_2O_2 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 signal. Another important enzyme used in immunohistochemistry is *alkaline phosphatase* (AP). In this system, endogenous enzyme activity is blocked by the addition of levamisole to the substrate medium. This is necessary especially in gut and placenta, where a high level of endogenous alkaline phosphatase activity is 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 [13–15]. 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 non-diffusible, permanent staining and sections can be counterstained with routine methods and mounted in xylene-based mountains [8, 16, 17] (Fig. 1.2). The intense reaction makes the immunogold method valuable for morphometric studies using image analysis equipment. Since 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 kidneys, gut and liver [18].

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 peroxydase-anti-peroxydase (PAP) – or alkaline –phosphatase – anti-alkaline – phosphatase (APAAP) technique, which is based on immunological binding affinities, and (2) the avidin–biotin-complex (ABC) technique, which is based on chemical affinity. The *PAP* [19] and *APAAP* systems [20] make use of a soluble immuno-complex, which 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 which forms the immuno-complex. 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 [21] 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. 1.3). Also, in this method, a link antibody, which is covalently bound to biotin has to be applied. There is a potential problem of avidin binding to endogenous biotin particularly in liver and kidneys as well as in frozen sections. It can be reduced by preabsorption of the tissue sections in avidin followed by biotin.

For the different enzymatic labels, several chromogens are available. The most widely used chromogens among

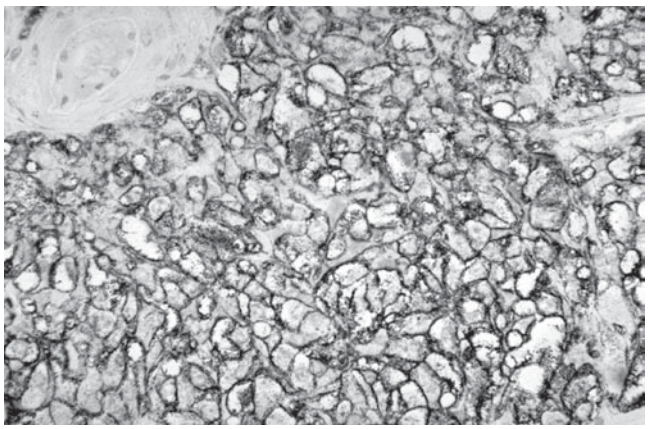

Fig. 1.2 Immunohistochemical detection of polysialic acid in a medullary thyroid carcinoma using immunogold technique with photochemical silver amplification. Note the strong cell membrane staining of tumor cells

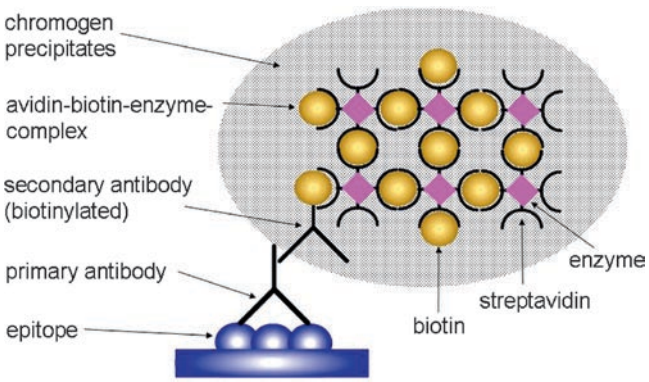

Fig. 1.3 Schematic representation of the ABC method

others include DAB (brown) (Fig. 1.4) and AEC (red) for the detection of HRP, and, fast red or new fuchsin for AP activity. When long incubation times for AP substrates are required (e.g., in non-radioactive, immuno-based detection systems of in situ hybridization with digoxigenin-labeled probes) the chromogen 5-bromo-4-chloro-indoxylphosphate-tetrazolium (NBT-BCIP) is used [22].

1.2.5 Signal Intensification and Amplification

In recent years, several attempts have been undertaken to further increase 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 [23]. The *nickel–cobalt intensification* procedure converts the brown DAB precipitate in a black reaction product that is easily visible with high contrast in tissue sections or cells (Fig. 1.5). The technique described by Adams [23] 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 alliance can also successfully be applied to double immunostaining procedures. Another recently introduced modification of immunohistochemistry is the biotin amplification technique

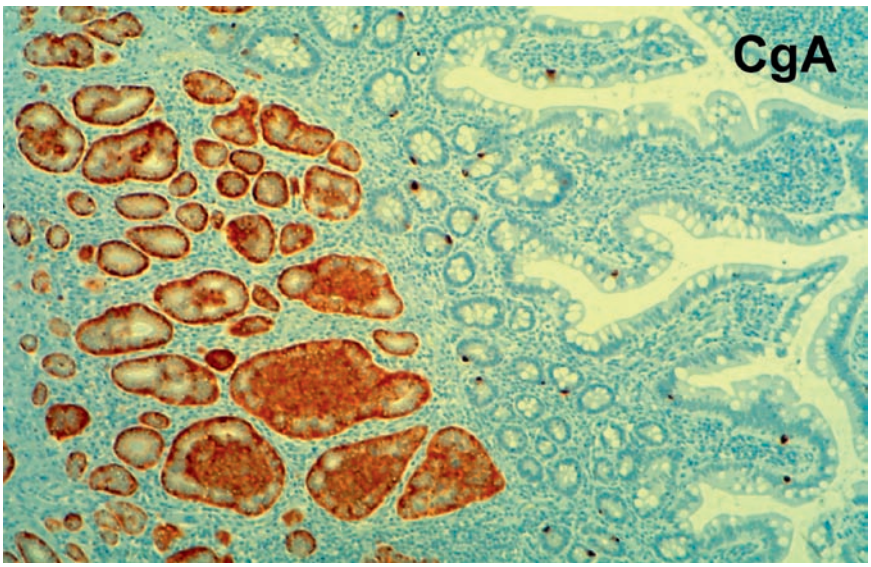

Fig. 1.4 Immunohistochemical detection of chromogranin A in a well differentiated neuroendocrine tumor (carcinoid) of the terminal ileum using the ABC method. Note the labeling of endocrine cells in the non-neoplastic mucosa

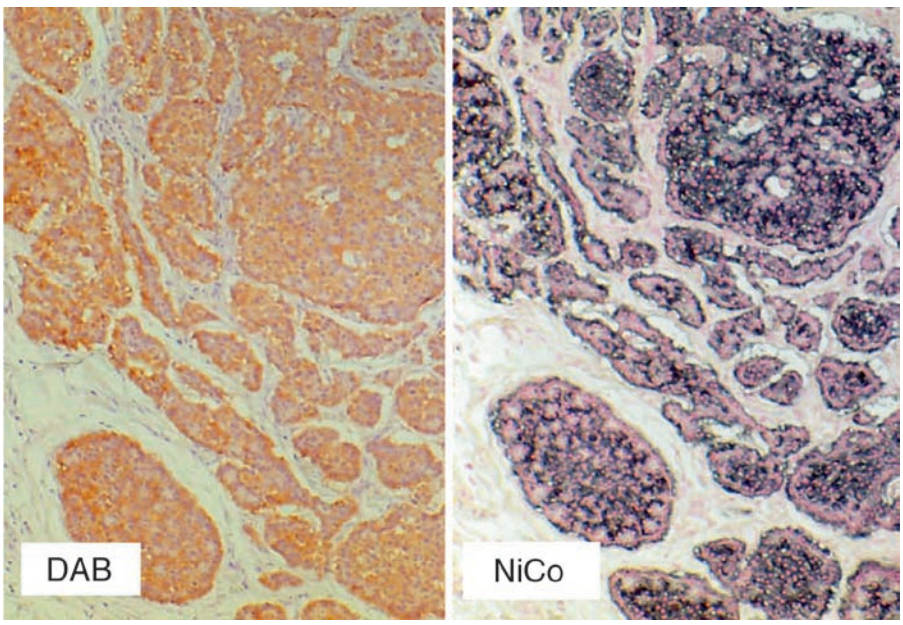

Fig. 1.5 Immunohistochemical detection of chromogranin A in a well differentiated neuroendocrine tumor (carcinoid) of the terminal ileum using the ABC method with DAB as chromogen (*left panel*; DAB) and nickel–cobalt intensification of the DAB signal (*right panel*; NiCo)

called *catalyzed reporter deposition (CARD)* method [24, 25] (Fig. 1.6). In our hands, CARD procedure allows a significant increase in sensitivity of immunohistochemical signals compared to conventional ABC-procedures without production of increased background [11] (Fig. 1.7). This amplification technique, which has also been applied for in situ hybridization [26–29] 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 e.g., streptavidin–biotin-complexes or enzyme/fluorochrome-labeled streptavidin [11].

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*) [30] and two-step system (*Envision*). In both systems, primary or secondary reporter molecules are directly bound to a large polymer which allows for the rapid one-or two-step detection of epitopes without significant background staining.

1.2.6 Double and Triple Labeling

The demonstration of two or more antigens in the same tissue section or cell can be achieved either by 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

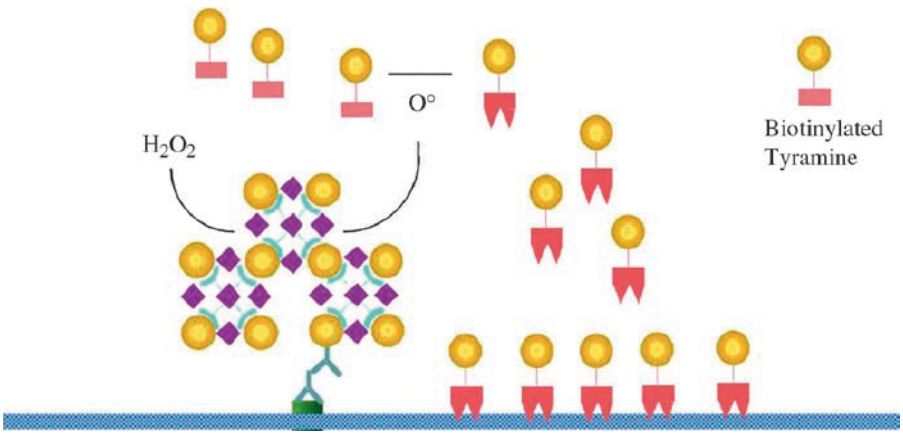

Fig. 1.6 Principle of catalyzed reporter deposition (CARD) using biotinylated tyramine

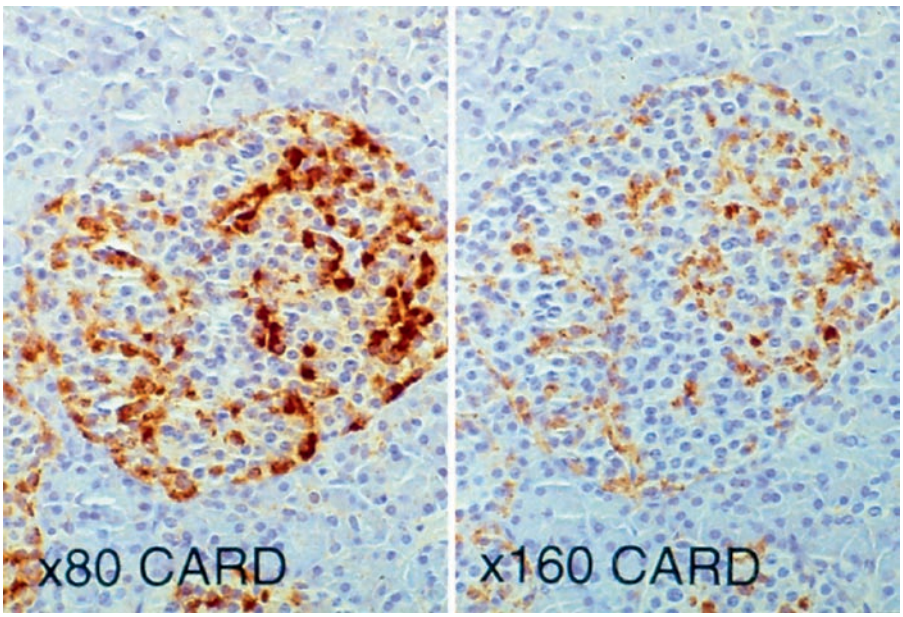

Fig. 1.7 Immunohistochemical detection of glucagon in a pancreatic islet using CARD signal amplification. After 80× dilution of the primary antibody (*left panel*) there is still a strong signal detectable, which is slightly reduced after 160× dilution of the primary antibody (*right panel*)

more enzyme systems and (3) a combination of enzyme systems and immunogold labeling (Fig. 1.8).

Ideally, primary antibodies are raised from different species in order to prevent cross-reactivity. If not possible, the use of two different enzyme labels or the combinations of enzyme and immunogold or fluorescent systems have to be applied [31]. Triple immunostainings are also available e.g., using an immunogold system for the third immunoreaction.

1.2.7 Controls and Testing of Antibodies

In order 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 non-immune serum from the same 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 e.g., in situ hybridization, western blot. Furthermore, positive and negative control samples (tissue with known positivity and negativity for the appropriate antibody used), which 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, since built-in controls (such as normal islets and exocrine tissue adjacent to endocrine pancreatic tumors) may serve as appropriate "internal" positive and negative controls. More recently, the application of control tissues on the same glass slide as the investigated tissue section has been described. This approach allows to run more slides e.g., in an automated immunostainer at the same time and allows storage of the control together with the diagnostic reaction.

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 1.1.

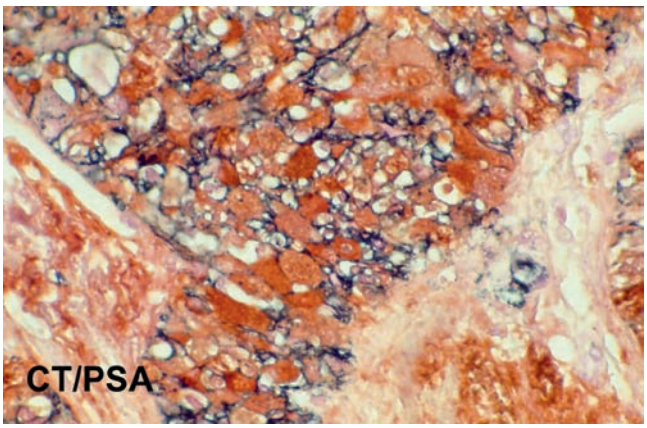

Fig. 1.8 Double immunolabeling of calcitonin (CT) and polysialic acid (PSA) in a medullary thyroid carcinoma. Note the cytoplasmic brown staining of calcitonin in the tumor cells and the black cell membrane signal of polysialic acid

1.2.8 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, its hyperplasias as well as neoplasias [32]. The WHO-classification also includes immunohistochemical results of hormone expression as well as proliferation activity of tumors [33, 34].

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 1.2) [9].

Table 1.1 Approach for testing new antibodies

Antibody dilution	Epitope retrieval	Blocking steps	Immunohistochemical detection
1. Polyclonal 1:100… monoclonal 1:10…	None Trypsin 0.01% Pronase 0.1%	None Defatted milk 2–4%	ABC-System, DAB
2. Increase/decrease dilution	Microwave 20 min in monohydrate citric buffer 0.01 M, pH 6.0	BSA 1%	Nickel–Cobalt amplification
3.		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)	

Table 1.2 Immunohistochemical neuroendocrine markers*

Markers for neuroendocrine cells (diffuse neuroendocrine system)	
Broad-spectrum markers	Chromogranins (CgA)
	Synaptophysin (Syn)
	Neuron-specific enolase (NSE)
	PGP9.5
	Leu7
	Synaptic proteins (SNAP-25, Rab3a)
	Neural cell adhesion molecule (NCAM), polysialic acid
Specific markers	
Adrenal medulla	Catecholamines, enkephalin, S-100 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, alpha-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, alpha-chain of glycoproteins, Ilet-1
Paraganglia	Catecholamines, enkephalin, S-100 protein, somatostatin, VIP, keratin (some)
Parathyroid	Parathyroid hormone (PTH), parathyroid hormone-related protein (PTH-RP)
Pituitary	ACTH, growth-hormone, FSH, LH, prolactin, TSH, alpha-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, melan A
Gonads (steroid producing cells)	Inhibin, steroid-metabolizing enzymes, vimentin
Thyroid (follicular cells)	Thyroglobulin, keratin, thyroid transcription factor-1 (TTF1)

*Partly adapted from [41, 42]

VIP vasoactive intestinal polypeptide; *ACTH* adrenocorticotropic hormone; *FSH* follicle stimulating hormone; *LH* luteinizing hormone; *TSH* thyroid stimulating hormone; *PP* pancreatic polypeptide; *D11* adrenocortical marker [40]; *SF1* steroidogenic factor 1; *CEA* carcinoembryonic antigen

The most widely applied *broad-spectrum neuroendocrine markers* are chromogranin-A and synaptophysin. *Chromogranin-A* is present in the majority of neuroendocrine cells and neoplasms; however, tumors which are degranulated or contain only a small number of secretory granules (e.g., small cell carcinoma of the lung and Merkel cell carcinomas) usually only exhibit a weak immunoreactivity. *Synaptophysin* is a 38-kDa protein, which is present in the membrane of presynaptic small vesicles. Since its immunoreactivity is independent of the presence of secretory granules, it usually exhibits a higher sensitivity than the marker chromogranin-A. *Neuron-specific enolase* is another broad-spectrum marker, which is not very specific since it also reacts with some non-neuroendocrine cells and tumors. But it can be useful in combination with the previously mentioned broad-spectrum markers, due 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, which is expressed in neuroendocrine cells, is the neural cell adhesion molecule NCAM (CD56) and its polysialylated form (which is detected by polysialic acid immunoreactivity) [31, 35, 36].

Neuroendocrine cells and tumors can be further characterized by the expression of *specific markers* listed in Table 1.2, as well as *proliferation markers* such as MiB-1 and *endothelial markers* such as CD31 (to quantify the proliferation index and visualize angioinvasion, both being important criteria in the new WHO-classification) [34, 37]. 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 [38]. 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, in situ hybridization to detect *mRNA* can be helpful.

Despite the above-mentioned limitations, immunohistochemical profiles can be helpful to narrow down possible primary neuroendocrine tumors [39–43], to distinguish benign from malignant endocrine lesions [37, 44, 45] or to provide prognostic and therapeutic information [46–55]. It is also an important tool in research e.g., to verify overexpression of oncogenes and loss of tumor-suppressor genes in endocrine neoplasms [56, 57].

1.3 Molecular Methods

1.3.1 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 exit four types of bases: *adenine*, *thymine*, *guanosine* and *cytosine* (A, T, G, C). In RNA, *uracil* (U) substitutes T (Fig. 1.9).

DNA and RNA also differ in the identity of the sugar, with desoxiribose present in DNA and ribose in RNA. DNA is a polymer of nucleotides linked together by phosphodiester-bonds between carbon 5 of one sugar and carbon 3 of the following sugar (Fig. 1.10). Nucleotide sequences of a polynucleotide are always read from the 5′ to the 3′ direction, since 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 base pairs 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. 1.11). The two strands of DNA in a double-helix are oriented in opposite direction 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.

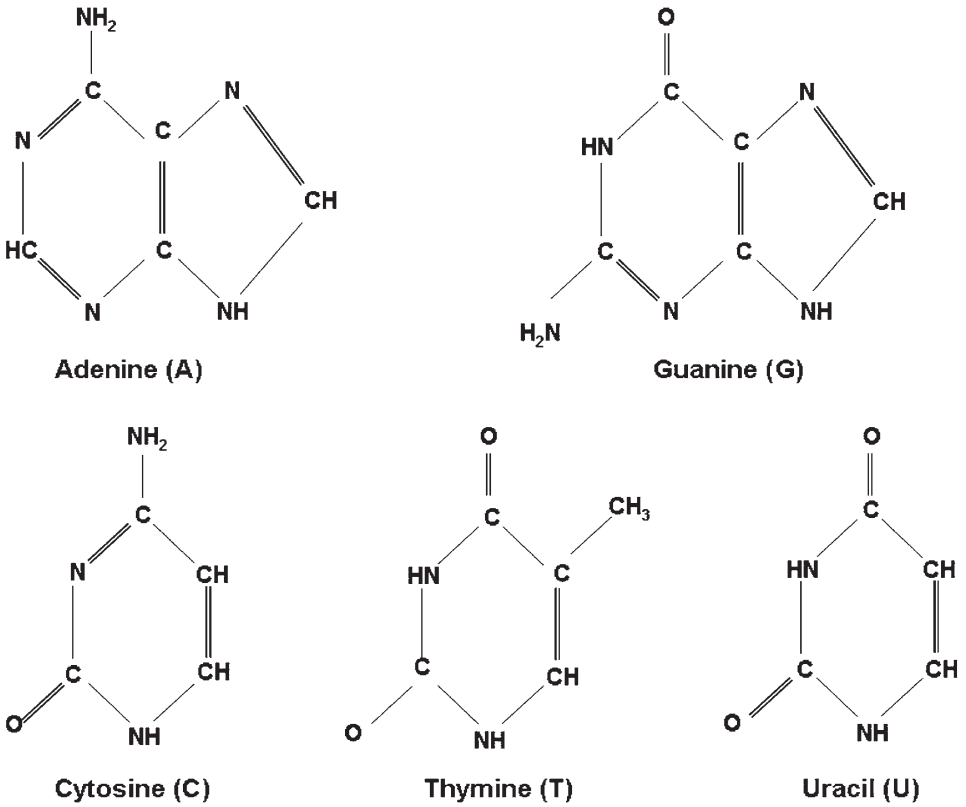

Fig. 1.9 Common bases found in nucleic acids

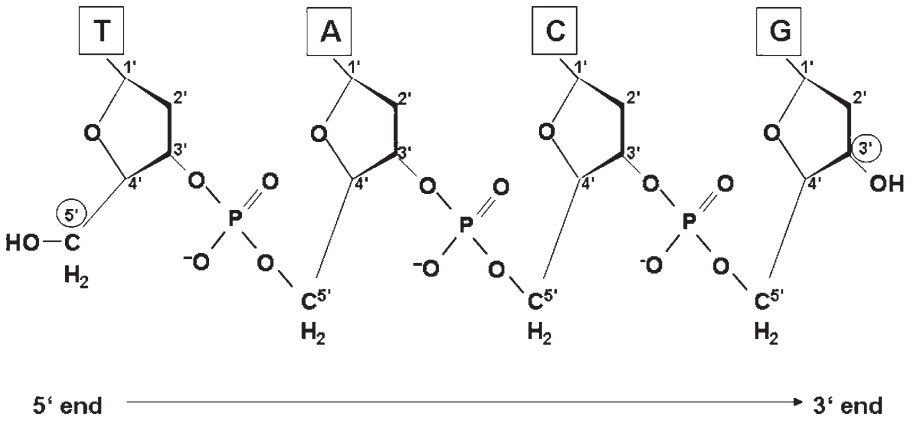

Fig. 1.10 Chain of nucleotides in DNA. Bases (T, A, C and G) are bound to desoxiribose molvecules at carbon atom 1′. Phosphodiester bonds link carbon atom number 3′ with number 5′ of the successive sugar residues. The so called 5′ end of the DNA strand has a terminal sugar residue in which carbon 5′ is not linked to a neighboring sugar and the 3′ end one with an unbound carbon 3′ (5′→3′ direction of DNA)

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 approximately 30,000–40,000 functional genes.

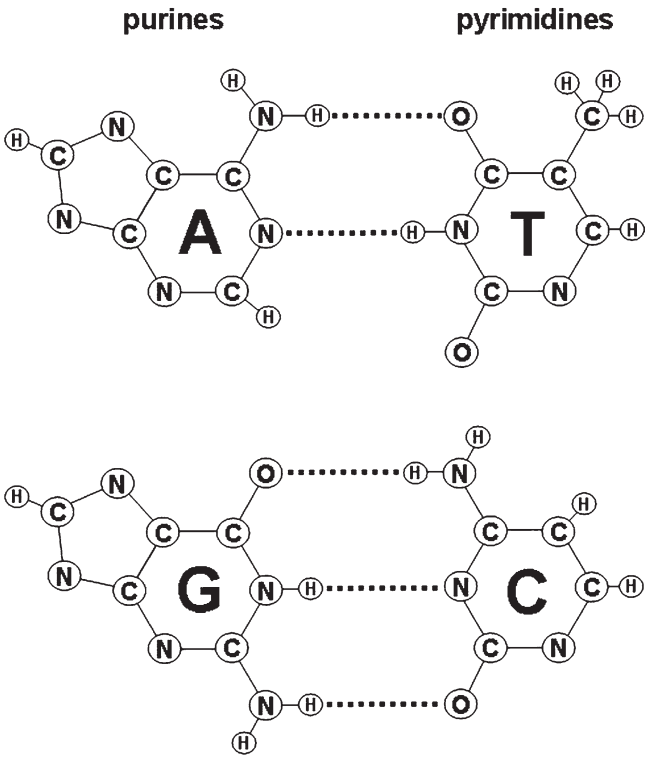

Fig. 1.11 Hydrogen bonding between matching base pairs. There are two bonds between adenine (A) and thymine (T) and three bonds between the guanine (G) and cytosine (C)

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. 1.12). It is synthesized in 5′ to 3′ direction by RNA polymerases. Within a gene, the information for the amino-acid sequence (the *coding sequence*) is contained in gene units called exons, which are interspersed with non-coding sequence units, termed *introns*. The entire portion of the DNA (including introns and exons) is transcribed into pre- messenger RNA (pre-mRNA). This precursor is rapidly 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 3-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. 1.13) and protein synthesis is initiated by the methionine codon AUG (start codon) and terminated by the stop codons UAA, UAG, and UGA (Fig. 1.12). Once translated, precursor protein products are further processed to form the mature gene product, a process that is termed *posttranslational modification* (Fig. 1.12). A single gene can be responsible for the generation of more than one

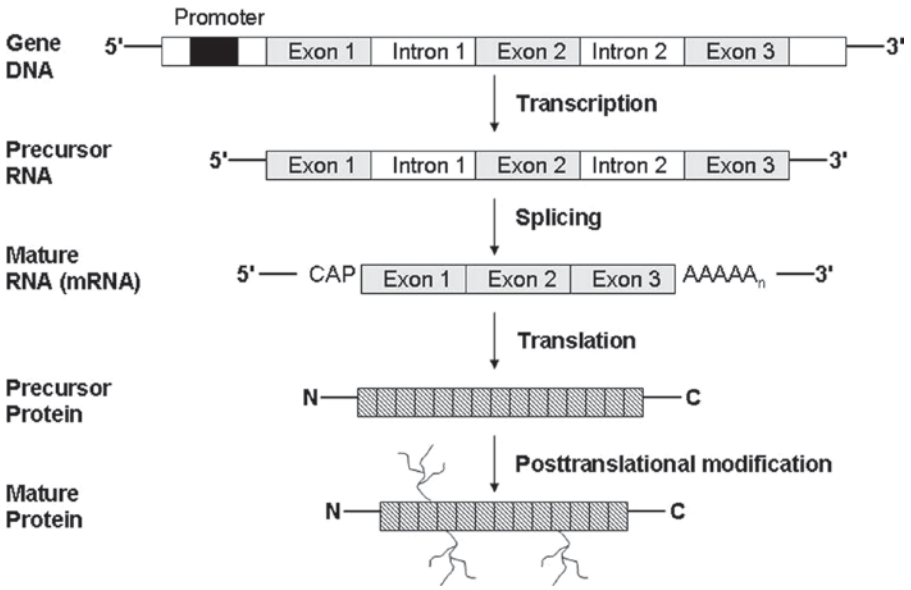

Fig. 1.12 Pathway of gene expression from the gene to protein product in an eukaryotic cell

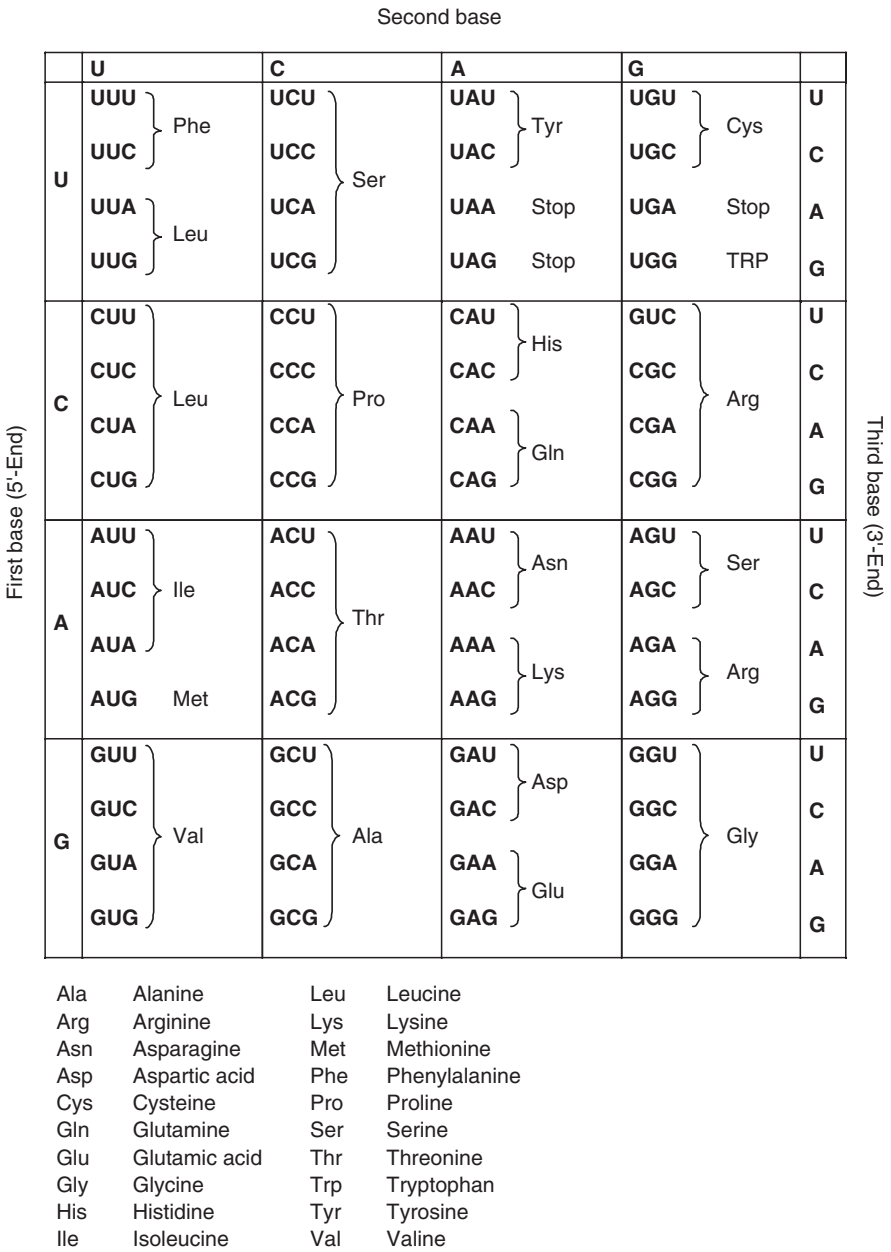

Fig. 1.13 The nuclear genetic code

specific mRNA because of alternate splicing of the precursor RNA so that the mature mRNA represents only a part of the original gene transcript.

1.3.2 In Situ Methods

1.3.2.1 In Situ Hybridization

In situ Hybridization (ISH) is a technique, which 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 [58]. In more recent years, ISH has also been applied to the localization of viral DNA sequences, mRNA and chromosomal mapping [22, 59, 60]. Furthermore, techniques have been adapted for ultrastructural analysis using electron microscopy [61].

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 forming stable hybrids (Fig. 1.14). 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. 1.15). 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 issues such as laboratory equipment, availability of reagents and the training of personnel.

Double-stranded DNA probes have found widespread application due 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. Double-stranded DNA-probes, which have to be denatured before use, can be generated by nick translation, random priming or the polymerase chain reaction (PCR) in the presence of labeled nucleotides (Table 1.3). *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.

Fig. 1.14 Principles of in situ hybridization. A labeled probe binds to the matching complementary sequence of the DNA

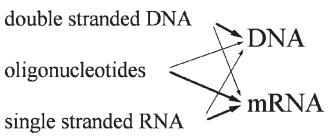

Fig. 1.15 Probe types and possible targets for in situ hybridization

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 of 20–50 basis 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 a 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, etc. 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, however, 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 [22] (Table 1.3).

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. 1.16). 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 further be

Table 1.3 Labeling methods for ISH probes

ds DNA	Random priming
	Nick translation
	PCR
Oligonucleotides	5′-end labeling
	3′-end "tailing"
cRNA	In vitro transcription

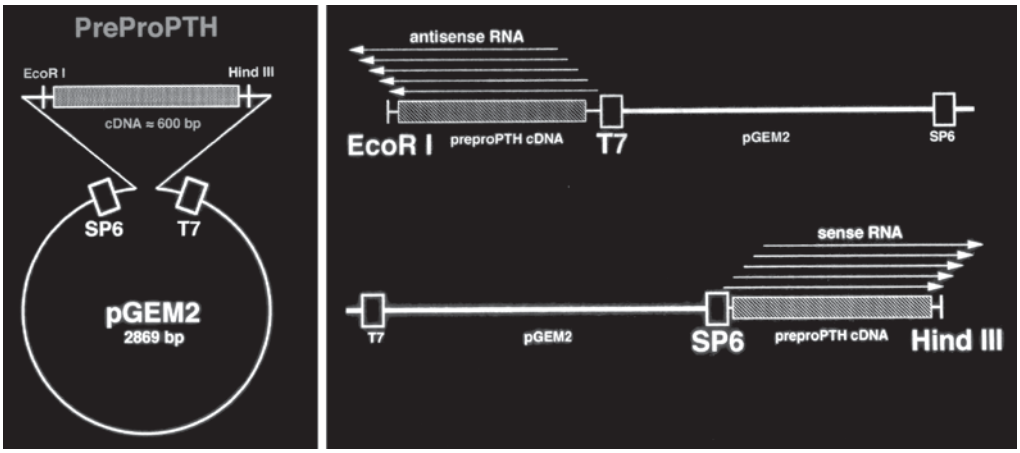

Fig. 1.16 *Left:* pGEM2 expression vector with preproPTH cDNA cloned into the polylinker site (EcoR I and Hind III restriction sites). *Right:* Schematic representation of antisense and sense RNA probe synthesis using in vitro run-off transcription with the linearized plasmid and the appropriate RNA polymerase (T7 for antisense and SP6 for sense RNA)

used for in vitro transcription without knowledge of cloning techniques [62]. The synthesized RNA can be labeled during synthesis by incorporation of ribonucleotides with coupled reporter molecules (in vitro "run off" transcription) (Table 1.3). The advantages of antisense RNA probes include a constant defined probe size, a high specific activity, a high thermal stability of RNA/RNA-hybrids and the possibility to generate sense strand (control)-probes. Furthermore, competitive hybridization to the complementary strand, as it occurs with double-stranded DNA probes, is excluded and non-hybridized (single-stranded) probes can be removed using RNAse digestion after hybridization [22, 60].

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) non-isotopic labeling using biotin, digoxigenin, fluorescein, alkaline phosphatase, 5-bromodeoxyuridine and others (Table 1.4).

Radioactively labeled probes, as originally described by Gall and Pardue [63], are still applied for in situ hybridization because of several reasons: (1) efficiency of probe synthesis can be monitored by scintillation counters, (2) radio-isotopes 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 [22, 64]. However, sections hybridized with 3H-labeled probes, usually require a rather long exposure (weeks) for signal 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.

Table 1.4 Probe labels

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

The use of radioisotopes for in situ hybridization 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 [65]. Furthermore, in situ hybridization with isotopic probes is difficult to perform on a routine basis in a non-specialized clinical laboratory, particularly when analysis must be performed frequently and results obtained quickly.

Non-radioactive probes are much more stable, safer in use, and provide a superior resolution of signal detection together with shorter turn-around times of procedure. Furthermore, the variety of available non-radioactive probe labeling systems provides the opportunity to detect different nucleic acid sequences simultaneously in the same tissue. Visualization of non-isotopic 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* [65]. 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 in situ hybridization and 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 revel 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. Consequentially, 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 in situ hybridization 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 in situ hybridization 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 in order to increase probe penetration, particularly in paraffin sections. Commonly, proteinase K or self-digested pronase is used [22]. Alternative methods are microwaving sections in citrate buffer [11, 66]. 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 acetylation step with acetic anhydride to reduce non-specific 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 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 non-specifically.

Hybridization

One of the important advantages of in situ hybridization 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 used probe, 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* (*Tm*) 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 over 90% homology [67]. There exist several formulas for calculating Tm and for e.g., oligonucleotide probes, the following formula can be used:

Tm = 81.5 + 16.6 log (molarity of monovalent cations = sodium concentration) + 0.41(% GC) − 675/L (length of probe in bases) − 0.62 (% formamide) − % mismatch [67].

RNA–RNA hybrids are generally approximately 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 non-radioactively 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 non-specific (e.g., salmon sperm) DNA and other bioactive polymers (to enhance binding and to reduce non-specific background) [22]. 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 non-specifically 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 non-specific background signals can further be reduced by treatment of the slides with ribonuclease A (RNAse), since the enzyme digests single-stranded, but not double-stranded RNA hybrids [64].

Visualization of Signal

For *radioactive probes*, signal detection is performed by *autoradiography*. Slides are dipped in liquid nuclear track emulsion (in the dark), which is then dried, exposed and

developed. Exposure is carried out at 4°C, since the efficiency of autoradiography is greater than at room temperature. After developing, the tissues are stained with a nuclear stain, mounted and coversliped (Fig. 1.17). 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. 1.18). The most common methods for visualizing *non-isotopic 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. 1.19) or immunogold (see Sect. 3) [22].

Controls

In order to ensure specific hybridization, a variety of controls have to be performed to detect false-positive and false-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), in situ hybridization without probe, competition studies with excess of unlabeled specific probe, hybridization with a non-specific (e.g., viral or vector) sequence or a sense probe, and the pretreatment of sections with RNAse or DNAse should be included.

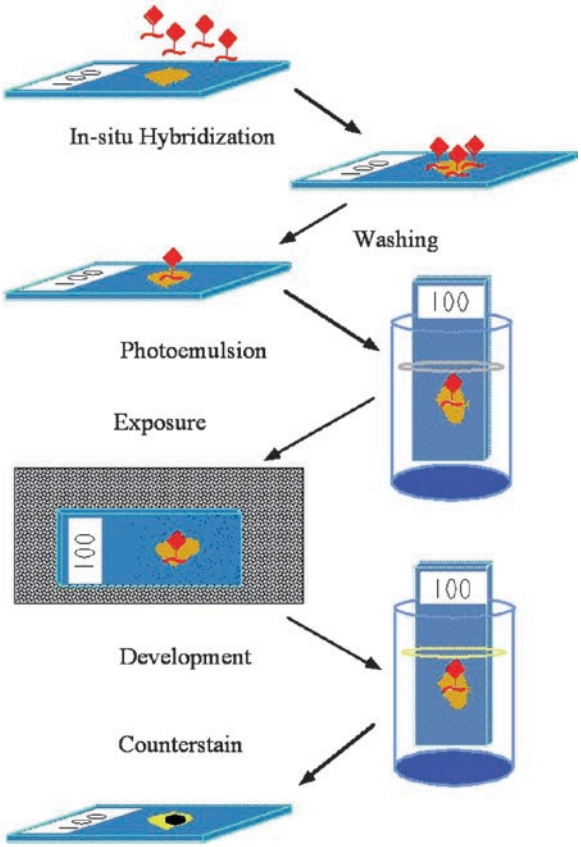

Fig. 1.17 Schematic representation of radioactive in situ hybridization

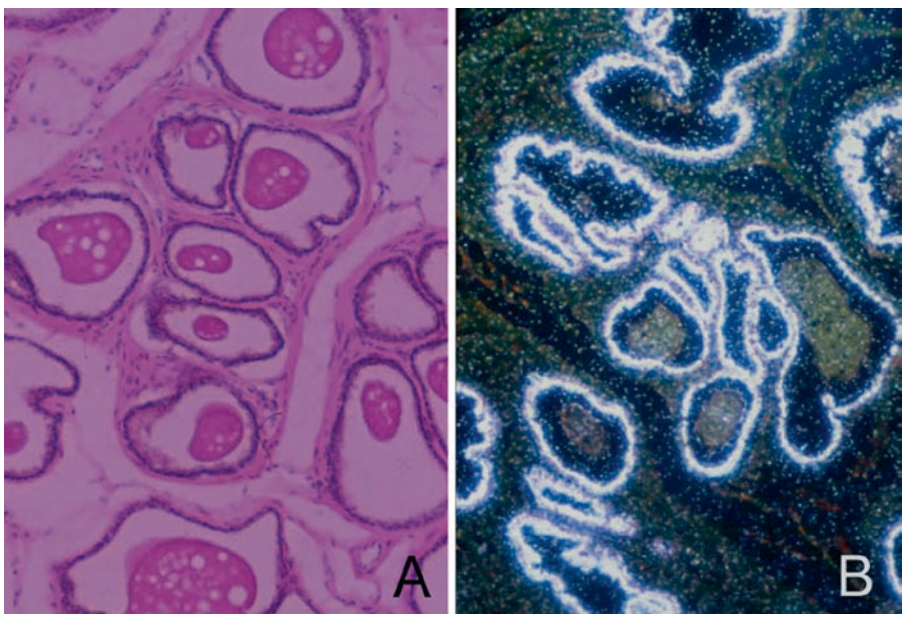

Fig. 1.18 Radioactive in situ hybridization using S^{35} labeled antisense RNA probes for the detection of SVS II in the rat prostate. (**a**) silver grains over the epithelium of the prostate epithelium are barely visible using bright field microscopy. (**b**) strong signals are detectable using *dark* field illumination

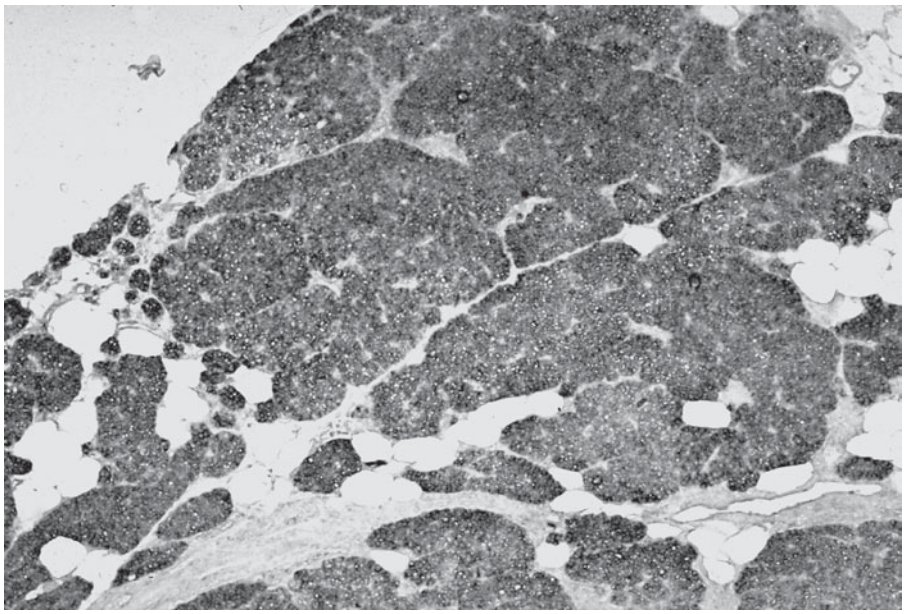

Fig. 1.19 Non-radioactive detection of PTH mRNA in a parathyroid gland using a digoxigenin-labeled preproPTH antisense RNA probe

1.3.2.2 Target and Signal Amplification

In order to detect low copies of nucleic acids in tissue sections and cells, several attempts have been made to increase the sensitivity of ISH procedures, such as mixtures of labeled, non-overlapping 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 [68], in situ polymerase chain reaction (PCR) and the Catalyzed Reporter Deposition (CARD) system.

Protocols for successful *in situ PCR* have been independently developed by several groups in the late 1980s [69–73]. The principal steps of in situ PCR are the following [74]: After 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 which 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 which have been incorporated into PCR products during thermal cycling (*direct in situ PCR*) [75].

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 [76, 77].

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 [78]. 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 [77, 79]. False-positive signals in direct in situ PCR mainly result from incorporation of labeled nucleotides into non-specific 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 non-specific PCR products by cDNA or DNA fragments ("endogenous priming") [75]. Diffusion of PCR products from the site of synthesis to neighboring template negative cells can also lead to false-positive results, a phenomenon which has been termed "diffusion artifact" [80, 81] (Fig. 1.20).

Low-amplification efficiency, poor reproducibility and difficulties in quantitation of results [75, 80] have led to a more realistic attitude about the practical potential of in situ

PCR in recent years [82] and other approaches to target amplification such as *in situ self-sustained sequence replication (3SR)* [83, 84] and *in situ transcription* [85] have not found a broader acceptance in the field of in situ visualization of mRNA. The need for the above-mentioned target amplification techniques has significantly diminished since more refined microdissection and amplification methods are now available to identify DNA or RNA from small cell groups or even single cells harvested from tissue sections.

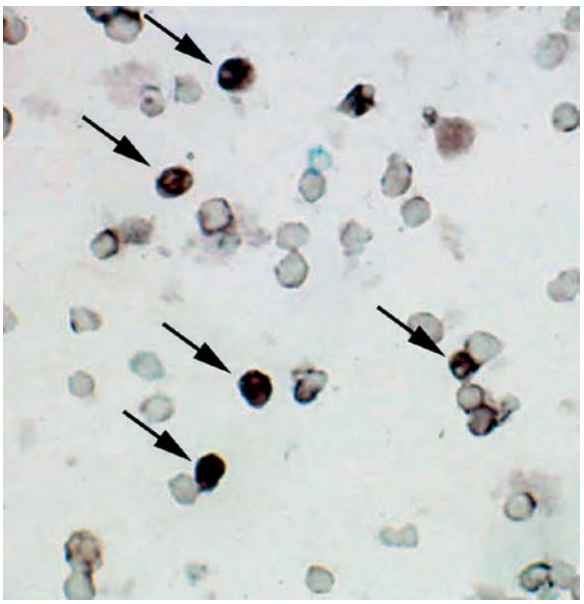

Fig. 1.20 Non-radioactive indirect in situ PCR for the detection of a rearranged VH3 DNA sequence in a mixture of rearrangement positive and negative clonal B cells. Note the strong nuclear signal (*arrows*) in rearrangement positive cells and the weak cytoplasmic signal in rearrangement negative cells (possibly caused by so called diffusion artifacts)

Recently, the CARD technique using biotinylated tyramine (tyramide) [24], has successfully been adapted to in situ hybridization in cytospins and tissue sections [27–29] as well as to FISH in metaphase and interphase preparations [86]. This signal-amplification technique, which has already been discussed for immunohistochemistry, can significantly increase the sensitivity of DNA and mRNA in situ hybridization. It appears to be more reliable than the above-mentioned target amplification methods [87] (Fig. 1.21). It can also be applied on routinely fixed, paraffin-embedded sections and the entire in situ hybridization procedure can be shortened to one working day (Fig. 1.22). We could demonstrate that tyramide conjugates such as Digoxigenin-, Biotin-, DNP-, TNP-, or fluorescein-tyramides [88, 89] provide approximately the same sensitivity, indicating that signal amplification is independent of the tyramide conjugate used [28, 29]. 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.

1.3.2.3 Applications of In Situ Hybridization in Endocrine Pathology

ISH has significantly advanced the study of gene structure and expression at the level of individual cells in complex structural tissues [90–92]. It has contributed substantially to the diagnosis and understanding of neoplastic endocrine diseases [93] and has provided invaluable insights onto hormone regulation, storage and secretion [94, 95]. 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

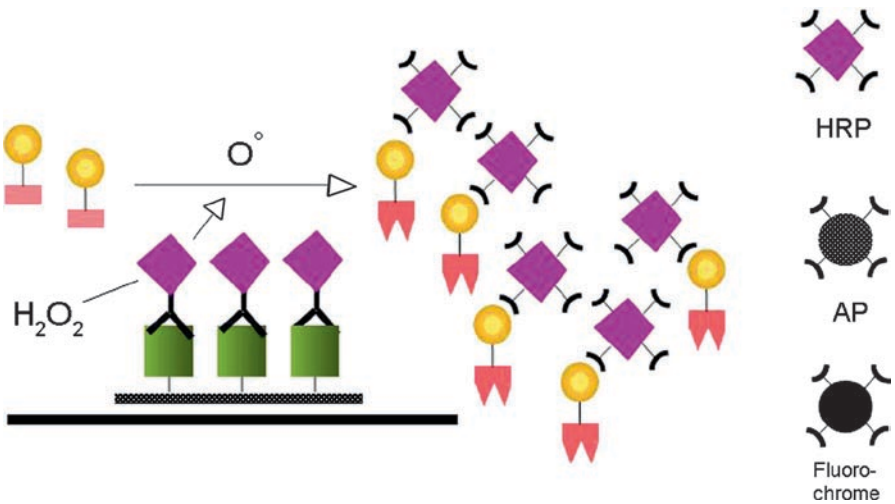

Fig. 1.21 Principles of in situ hybridization with CARD signal amplification using digoxigenin labeled probes and biotinylated tyramides. Horseradish peroxidase (HRP), alkaline phosphatase (AP) or fluorochrome labeled streptavidin can be used for visualization

modifications of hormones [96, 97], (2) effective antisera for immunohistochemistry are lacking or (3) it is not sure whether endo- or pinocytosis rather than specific gene expression is responsible for a positive immunohistochemical result [98]. Thus, ISH has been used to detect neuroendocrine genes in small cell lung carcinomas [99] and adrenocortical tumors [31], calcitonin-related peptide and calcitonin in medullary thyroid carcinoma [100, 101], preproparathormone in hyperplastic, adenomatous and carcinomatous human parathyroid glands [102] and specific hormones in pituitary neoplasms [103] (Figs. 1.19 and 1.22).

In some instances, the combination of in situ hybridization and immunohistochemistry is used to localize peptides and its mRNA simultaneously in a single section [104, 105] or alternatively in consecutive sections to provide evidence for the cell or tissue to be the site of synthesis [106–111]. For combined in situ hybridization and immunohistochemistry on the same slide, the in situ hybridization 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.

1.3.2.4 Fluorescence In Situ Hybridization

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. 1.23), 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

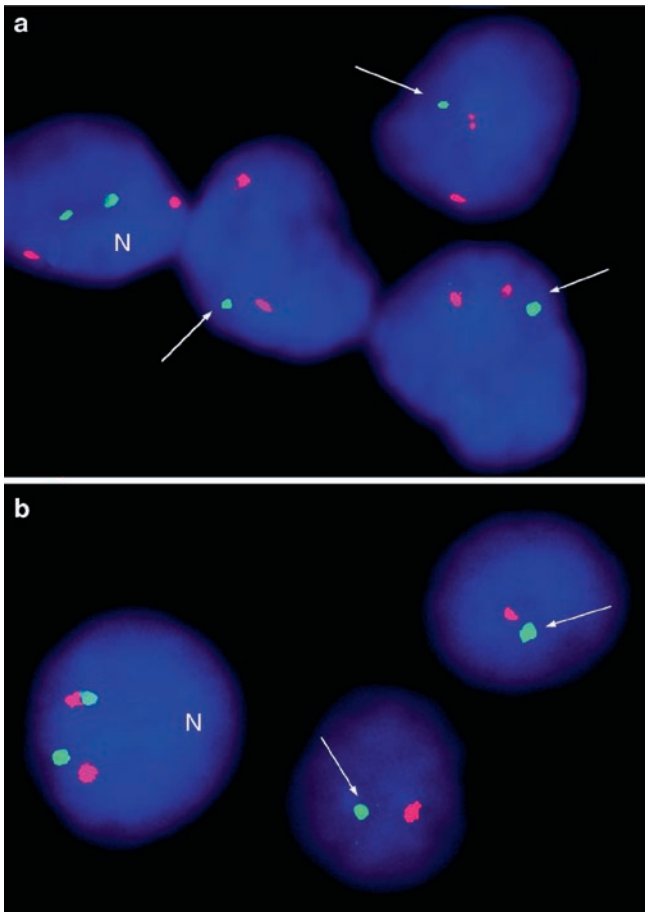

Fig. 1.23 Double FISH in interphase nuclei of two endocrine pancreatic tumors (**a** and **b**) using a centromeric chromosome 11 probe (*red* signal) and a MEN1 specific probe (*green* signal). In the tumors cells of A only one *green* signal is present (*arrows*) but two *red* signals are detectable (when compared to the normal cell; N), indicating a loss of one allele at 11q13. In the tumor cells of B one *green* and one *red* signal is missing (when compared to the normal cell; N), indicating the loss of one chromosome 11

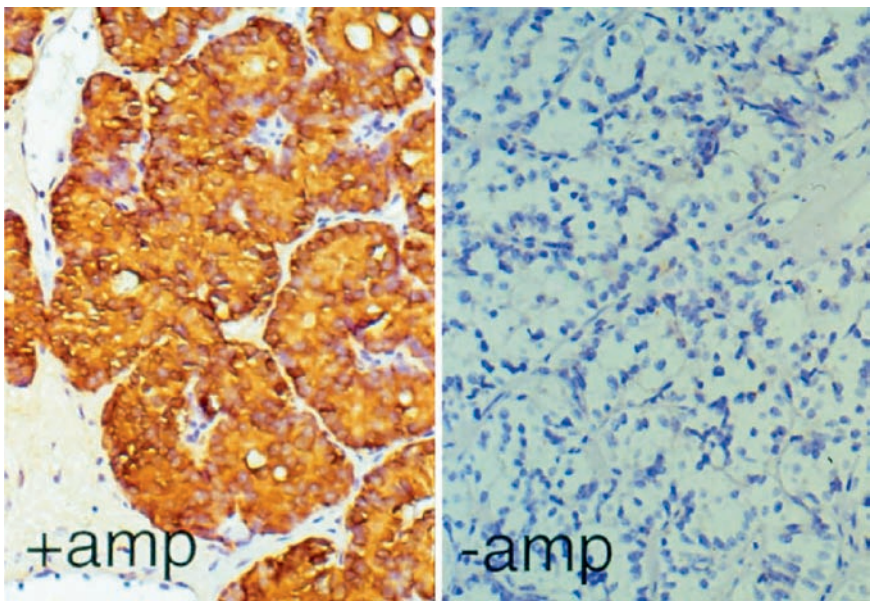

Fig. 1.22 In situ detection of insulin mRNA in an insulinoma using a highly diluted, digoxigenin-labeled insulin antisense oligonucleotide probe followed by CARD amplification for visualization (*left panel*; +amp) and without amplification (−amp). Note the strong cytoplasmic signal after CARD amplification in contrast to the very weak signal without amplification

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 fluorescent dyes with either a direct or indirect detection method, and the results can be evaluated by fluorescence microscopy using appropriate filters (Fig. 1.24).

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, e.g., 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 approximately 10–40 kb and BAC clones are more suited for those studies. Probes against single copy genes can be used for identifying *structural chromosome changes*, i.e., translocations, deletions, or gene amplifications (Fig. 1.23). 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 1.5.

Table 1.5 Selection of DNA-probes for the detection of chromosomes, distinct loci or genes related to endocrine neoplasias by FISH analysis

Gene/locus	Related to	References
X-, Y-chromosome Anomalies	Pancreatic endocrine tumors	[112]
3p25.3–p23 loss	Pancreatic endocrine tumors	[113]
9q34 gain	Pancreatic endocrine tumors	[114, 115]
6q22, 6q23–q24 loss	Pancreatic endocrine tumors	[116]
Chromosome 11 and MEN1 locus	MEN1 associated pancreatic endocrine microadenomas	[117]
RET rearrangements	Thyroid papillary microcarcinoma	[118]
RET rearrangements	Childhood papillary thyroid carcinoma	[119]
PAX8 PPARγ fusion	Follicular thyroid carcinoma	[120]
Trisomies of chromosomes 5, 8, and 12	Prolactinoma	[121]
Chromosome 11	Pituitary adenomas	[122]
Mutant RET allele in trisomy 10	Multiple endocrine neoplasia type 2-associated pheochromocytomas	[123]

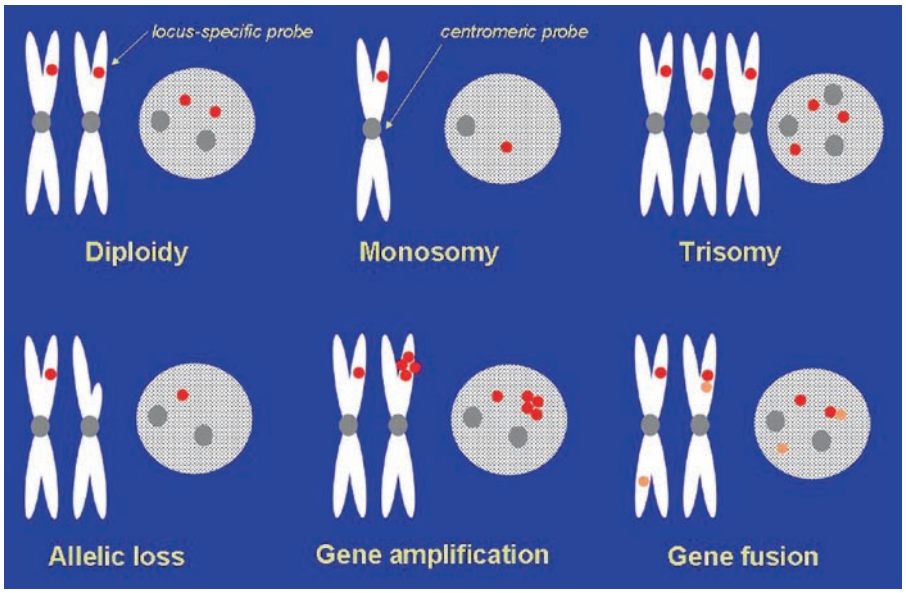

Fig. 1.24 Schematic representation of FISH results using centromeric (chromosome-specific) and locus-specific probes comparing metaphase spreads (*left*) and interphase nuclei (*right*)

1.3.2.5 In Situ Proteomics

Protein analysis has traditionally involved tissue homogenization, protein extraction (typically by multiple steps of liquid–liquid extraction and centrifugation), protein separation by liquid chromatography or gel electrophoresis, and molecular weight determination by mass spectrometry (MS) or gel electrophoresis. Unfortunately, this method suffers from many limitations, not the least of which is that once a tissue is homogenized, all spatial information is lost. Direct tissue analysis, however, makes it possible to localize proteins in a tissue section and, compared with multiple liquid–liquid extractions, minimizes analyte losses.

Matrix-assisted laser desorption/ionization (MALDI) imaging mass spectrometry (IMS) is a powerful tool for investigating the distribution of proteins and small molecules within biological systems through the in situ analysis of tissue sections. MALDI-IMS can determine the distribution of hundreds of unknown compounds in a single measurement and enables the acquisition of cellular expression profiles while maintaining the cellular and molecular integrity. In recent years, a great many advances in the practice of imaging mass spectrometry have taken place, making the technique more sensitive, robust, and ultimately useful.

Principle of MALD-IMS

The basic methodology of MALDI-IMS is simple: MALDI matrix (e.g., sinapinic acid) is applied to the tissue section, and the section is then analyzed by MS to allow the spatial, spectral composition to be plotted. Direct analysis of a tissue section using matrix-assisted laser desorption ionization mass spectrometry (MALDI MS) technology has already been shown to be a fast and effective means to view a window of many hundreds of protein signals over a wide molecular weight range [124, 125]. Many replicate analyses can be obtained from extremely small pieces of tissue because the laser spot size is typically about 50 μm in diameter. Each spot (or pixel) produced by irradiation of such a spot on tissue by the laser produces a spectrum of proteins desorbed just from that area. "Profiling" of that tissue section then may involve analysis of one or more spots from various areas of interest determined from histology. For a more complete information on the distribution of signals with the tissue, imaging of the tissue is done by analysis of an array of spots to give hundreds to thousands of pixels from a single biopsy specimen. One can display a mass spectrum for each pixel, covering proteins from molecular weight of a few thousands to >100,000. A plot of the relative intensity of any molecular weight species in each pixel over the area imaged thus produces a molecular weight–specific image of the tissue. Lasers operating at 1 kHz or faster can be used and so an analysis of ten spots on a biopsy where 200 laser shots are acquired per spot can be accomplished in few seconds, and data acquired from a target plate holding a hundred samples can be obtained in <10 min. Mass measurement accuracies are achievable below the 50–100 ppm (0.005–0.01%) range. Acquiring such data-rich spectral patterns necessitates advanced computational approaches to data mining and interpretation and represents a critical part of the process of discovery of protein signatures. It is important to note, that validations of several types are critical to ensure that data are correctly fitted and assignments made at high confidence. Through this process, molecular signatures can be discovered for a wide variety of clinically relevant questions. Hierarchical lists of protein molecular weights are produced that are the result of a given query followed by identification of the specific proteins involved using well-established MS methods. This often involves the use of electrospray ionization liquid chromatography tandem MS technology with fractionation of proteins from a tissue extract, protease digestion, peptide sequencing, and database-matching protocols [126].

Molecular Signatures from Tissues

A majority of MALDI-IMS studies have been dedicated to the study of proteins contained in human and animal tissue sections [124, 125]. Applications in the field of pathology hold particular interest for many because of the potential benefit for clinical diagnoses and treatment. Other studies have focused on proteomic events occurring in normally developing tissues. To date, profiling and imaging by MALDI-IMS has been applied to multiple diseased tissues, including human non-small-cell lung tumors [127], gliomas [128], breast cancer [129] and ovarian tumors [130] and continues to be a main focus of MALDI-IMS studies [131]. So far, no reports of MALDI-IMS on systematic series of endocrine tissues have been published. The application of MALDI-IMS have been reported on rat pituitary glands [132] and pancreatic islets [133]. In Fig. 1.25 MALDI-IMS of a tissue section of a rat pituitary gland is shown.

1.3.3 Liquid-Based Methods

1.3.3.1 Southern and Northern Blotting

Southern blotting is a technique for the analysis of double-stranded DNA molecules [134] and *Northern blotting* for RNA molecules by filter hybridization. Both methods are infrequently used in diagnostic endocrine pathology and occasionally applied for research purposes. Thus, for both techniques high molecular weight genomic DNA or total

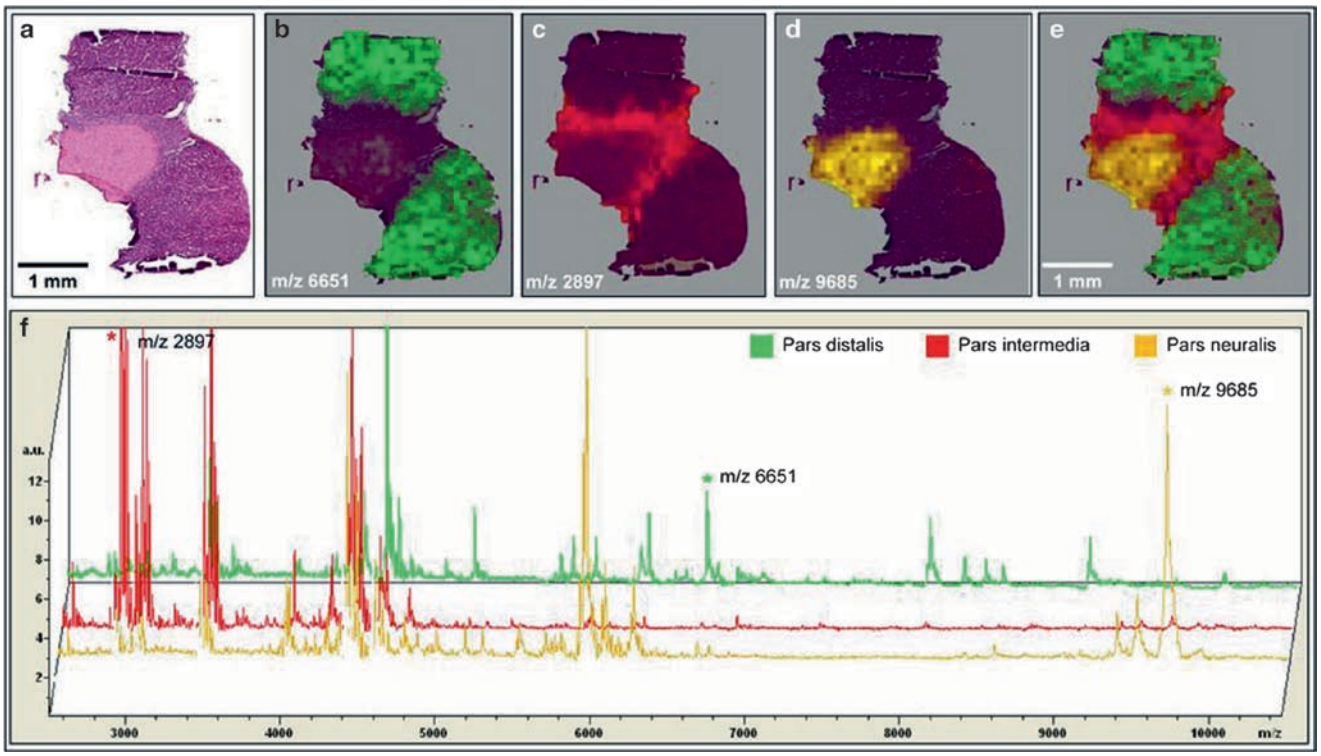

Fig. 1.25 MALDI-IMS of a tissue section of rat pituitary gland. (**a**) optical microscopic image of a H&E stained tissues section. The staining was done after the MALDI measurement of the tissue section. (**b**–**d**) visualized selected *m/z* species representing features to pars distalis (*m/z* 6,651; *green*), pars intermedia (*m/z* 2,897; *red*) and pars neuralis (*m/z* 9,685; *yellow*). (**e**) Merge of **a**–**d**. (**f**) MALDI-TOF MS spectra obtained from this case from pars distalis (*green*), pars intermedia (*red*) and pars neuralis (*yellow*) showing molecular differences between the histological regions. Scanning resolution 50 µm. *Scale bars* 1 mm

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 hydrolyze 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 the detection of specific sequences with a radioactively or non-radioactively labeled probe, which is capable of annealing to the DNA on the filter (see Sect. 1.3.2.1) (Fig. 1.26). 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. 1.27).

Northern blotting is the RNA-equivalent of Southern blotting. Because of the inherent differences between RNA and DNA, several modifications of protocols are required. Since RNA is single-stranded, no denaturation is required and due to the relative small size of messenger RNA, no restriction enzyme digestion is needed. Due 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 in situ hybridization.

1.3.3.2 Tissue Microdissection for DNA/RNA Extraction

Tissues are complicated three-dimensional structures, composed of different types of interacting cell populations. Since

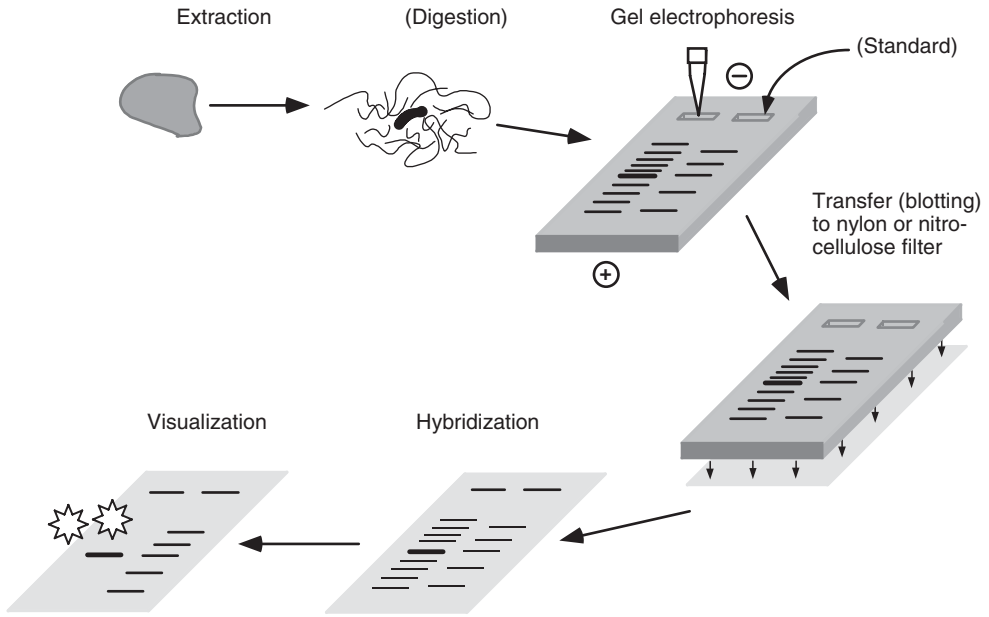

Fig. 1.26 Principle of blot hybridization

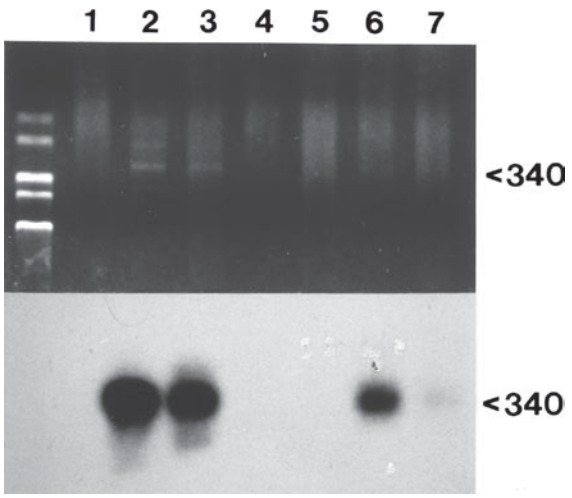

Fig. 1.27 Example of Southern blot hybridization to confirm specificity of PCR products using a digoxigenin labeled internal probe and chemoluminescence for visualization. Note that ethidium bromide staining of PCR products exhibits barely visible products of 340 bp in lanes 2, 3 and 6 and that strong signals are detectable after Southern blotting (*lower panel*)

the cell 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 versus diseased tissue. Thus, tissue microdissection represents one of the most promising techniques in molecular pathology, offering a link between morphology and molecular genetic analysis.

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. 1.28). An overview of the most common microdissection techniques in molecular pathology is given in Table 1.6. 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 non-coated glass slides. Manual tissue dissection requires histologically homogenous malignant lesions, and the areas should have a diameter of at least 1 mm [135]. Using a sterile needle or a scalpel, the selected lesions can be procured [136].

The principle of *laser cutting* is a locally restricted ablative photo decomposition process without heating the direct environment of the laser beam [137]. Within the diffraction limited focus of the laser beam obtained by a high-numerical

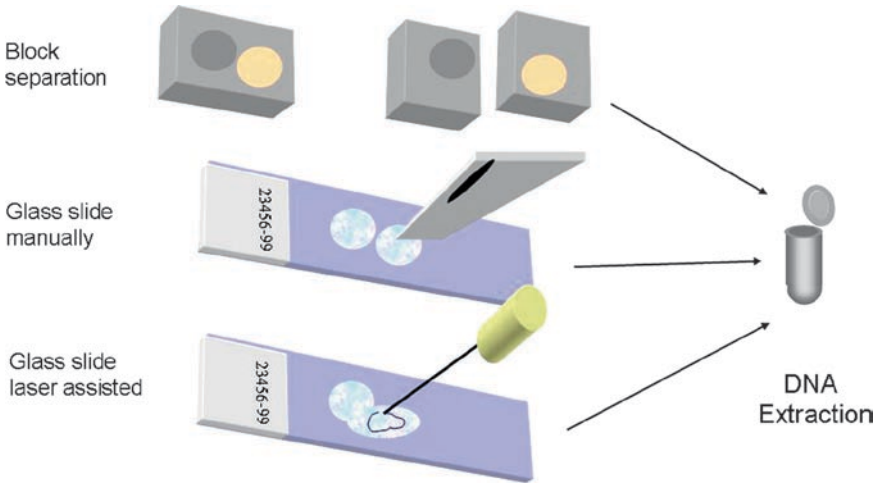

Fig. 1.28 Schematic representation of tissue microdissection

Table 1.6 Overview of the most common microdissection techniques in molecular pathology

	Manual tissue dissection	Laser microbeam microdissection (LMM)[a]	Laser pressure catapulting (LPC)[a]	Microdissection of membrane mounted tissue (MOMeNT)[a]	Laser capture microdissection (LCM)[a]
Function principle	Procurement of large tissue areas using a sterile needle or a scalpels with/without an inverted microscope	"Cold ablation" of unwant ed cells using a UV laser (337 nm)Procurement of remaining cells with or 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 (980–1,064 nm)
Minimum sample size	~50–100 nm	<1 µm	<1 µm	1 µm	> 7 µm
Preferential spectrum of use	Large and homogeneous cell areas (>104 cells)	Small lesions (<50 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

[a]For immunostained samples are also automatic systems available

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 µs) heat transfer is avoided [138]. 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 [139]. At the site of laser exposure and ablation, no amplifiable material is left behind [140]. 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 to catapult an isolated cell or cell group of its surrounding with a single precisely aimed laser shot out [141]. The ejected dissectates are either caught on a small piece of cover glass, or directly catapulted in the cap of a common PCR tube. The greatest advantage of this method is the procurement of the material in a "non-contact" 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, which is attached to a slide by nail polish [142]. 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 1,000 µm in diameter (Fig. 1.29). 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 [143]. The MOMeNT technique implicates 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 application of this technique is wide, and it allows the fast procurement of , histologically, homogenous tissue areas or single cells [144]. 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. The focal spot of the melting laser cannot reach below 7 µm in diameter and there is no possibility to selectively destroy 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 (HE) are commonly used for tissue microdissection. Other histologic stains such as methyl green or nuclear fast red may also be used [145]. 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 [143, 146]. It is helpful to increase the histo- and cytomorphology by covering the stained sections with a thin layer of xylene or 2-propanol, 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 [147, 148]. 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 can be used for microdissection and subsequent PCR analysis, even after storage of several years [149]. Other cell preparations, e. g. 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

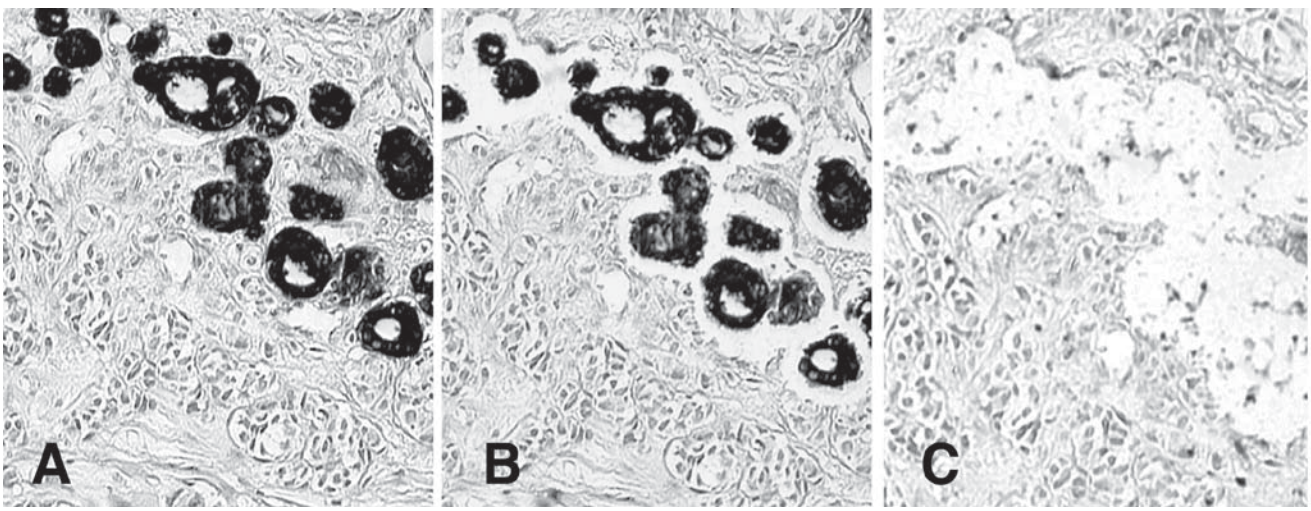

Fig. 1.29 Laser-assisted microdissection of thyroglobulin stained follicles (**a**) in a mixed medullary–follicular thyroid carcinoma. Follicles are isolated by laser (**b**) and catapulted into a Eppendorf tube for molecular analysis. (**c**) tissue section after removal of follicles

smaller samples. Thus, a simple one-step DNA preparation is recommended [140]. 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 can seemingly 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 RT-PCR amplification products than cross-linking fixatives such as formaldehyde [150]. However, we found no differences in the qualitative expression of several genes in formalin-fixed compared to fresh-frozen tissue [151]. For less than 10^5 cells, RNA amplification techniques should be applied.

1.3.3.3 Polymerase Chain Reaction

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 amplifying 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 result 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. 1.30 and 1.31).

Quantitative determination of DNA sequences and gene expression levels offers a powerful approach for the comparative analysis of normal and diseased, 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 (reverse transcriptase (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 for 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. 1.32). 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. 1.33). 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-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 [152]. Degenerate oligonucleotide primer PCR (DOP-PCR) provides a universal method for uniform amplification of small DNA samples [153]. The DOP-PCR procedure is useful as a first step in: In situ hybridization with flow-sorted chromosomes, comparative genome hybridization (CGH) or preparation of size-fractionated DNA fragments (e.g., for subtractive hybridization). Primers used for

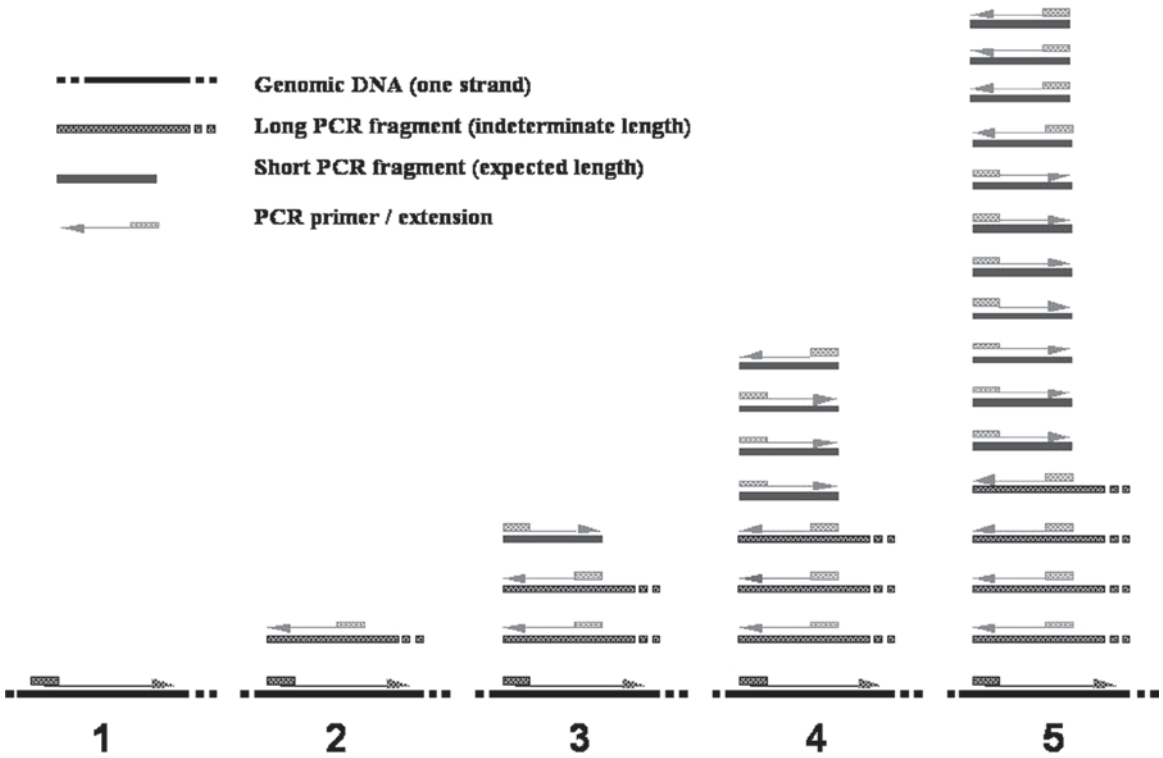

Fig. 1.30 Principles of polymerase chain reaction (one DNA strand). Note the linear amplification of PCR-products of intermediate length (flanked by one primer) and the exponential increase of numbers of PCR-products of expected length (flanked by both primers) after the second cycle

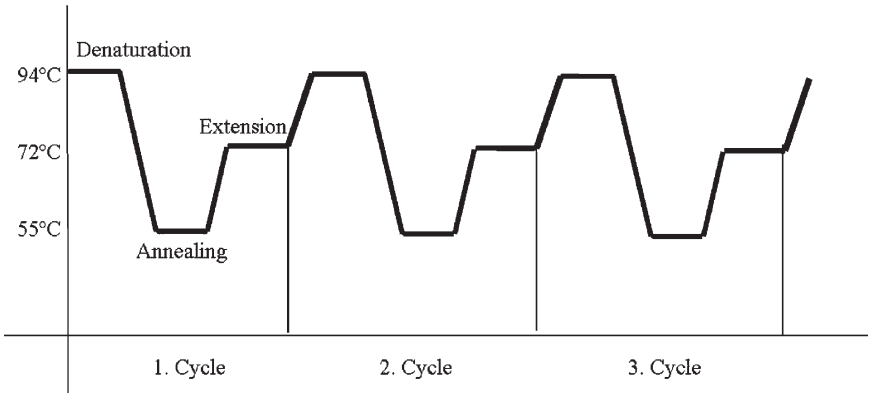

Fig. 1.31 Schematic representation of temperature cycling during PCR to yield DNA denaturation, primer annealing and primer extension

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. The next 35 cycles use a more stringent (higher) annealing temperature. Under the more stringent conditions, the material which was generated in the first five cycles is amplified preferentially, since 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.

Reverse Transcriptase-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 which 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. 1.32) [150, 154]. Specht et al. [154] 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.

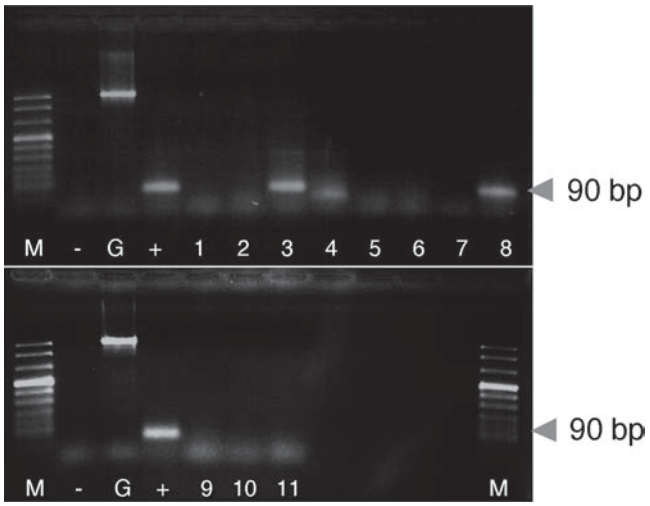

Fig. 1.32 Detection of *RET* mRNA expression in archival papillary thyroid carcinoma samples using reverse transcriptase PCR (RT-PCR). Note the positive signals in lanes 3, 4 and 8 (–: negative control; G: genomic control DNA; +: positive control)

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 (Fig. 1.33). The RT-PCR method is rarely used in diagnostics but more frequently applied for

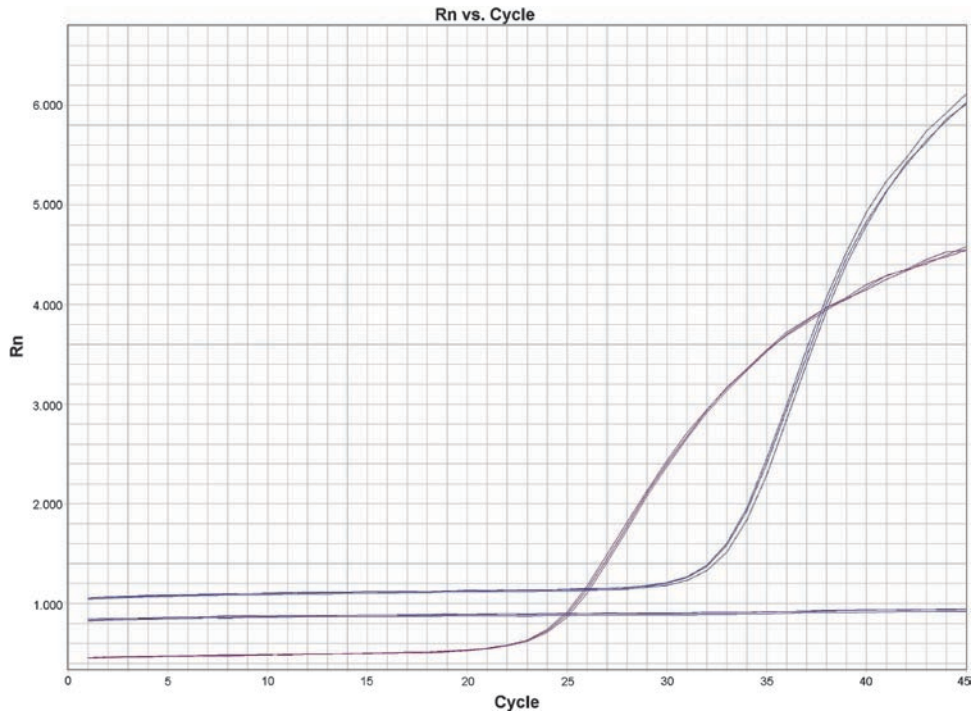

Fig. 1.33 "Real time" RT-PCR analysis of an archival tissue sample. The graph represents the detection of a reference and 2 marker genes (each in triplicate) via fluorescence monitoring of PCR products. During the early phases of the reaction (PCR cycle 0–18) no products are detectable (no fluorescence signals), whereas specific fluorescence signals, beginning at PCR cycle 21 and 23 onwards, indicate the presence of 2 gene-specific PCR products, whereas the second marker gene is not expressed

Table 1.7 Selection of recent applications of quantitative RT-PCR on endocrine tumors

Gene expression	Related to	References
Cyclooxygenase-2	Thyroid nodules	[155]
Kalpha1-tubulin	Thyroid anaplastic carcinoma	[156]
IGF II	Adrenocortical tumors	[157]
MYC, ERBB2, and CCND1	Malignant thyroid follicular cell tumors	[158]
Adrenomedullin, leptin, their receptors and neuropeptide Y	Hormone-secreting and non-functioning pituitary adenomas	[159]
Menin	Various adrenal tumors	[160]
Telomerase activity	Primary cultures of normal adrenocortical cells	[161]
Tgf beta	Insulinomas	[162]
Renin–angiotensin system	Pancreatic endocrine tumours	[163]

research purposes. Recent applications of quantitative RT-PCR in endocrine neoplasms are listed in Table 1.7.

1.3.3.4 Mutation Analysis

PCR-based methods are favored today for mutation analysis because they are easy to perform and require only low amounts of DNA. Different approaches to mutation analysis are needed in different situations. Screening methods as single strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE) or Denaturing high-performance liquid chromatography (DHPLC) are able to indicate a sequence difference compared to the wild type DNA. However, these methods can not 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 only cuts the mutant DNA strand (Fig. 1.34).

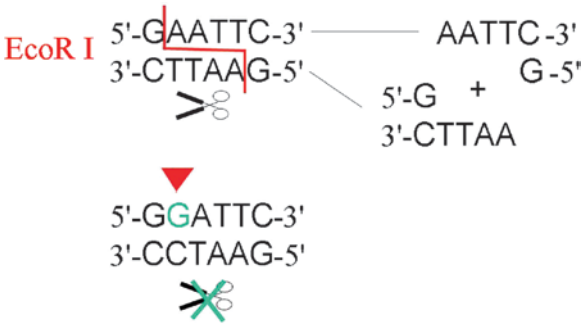

Fig. 1.34 Schematic representation of EcoR I restriction site. Loss of the restriction site by an A→G point mutation (*arrow head*)

DNA Sequencing

Today, *direct sequencing* is performed according to the dideoxy chain termination method described by Sanger [164]. 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 of the following nucleotide. Due to the mixture of normal and dideoxynucleotides, fragments of varying size are produced during the reaction. They start with the primer sequence and end for example with guanine, if dideoxy-guanine is added to the reaction (Fig. 1.35). 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 bottom to 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 dideoxynucleotides, all the 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 [165] (Fig. 1.36).

SSCP

The SSCPmethod is an efficient screening method for genetic alterations [166]. The PCR products are denatured by heating and then electrophoresed on a non-denaturing 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, silver [167, 168] 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 bee compared to the pattern of the wild type sequence. The additional bands can be cut out of the gel, subsequently be re-amplified and sequenced. SSCP is estimated to detect 70–95% of mutations in a fragment of 200 bp, the

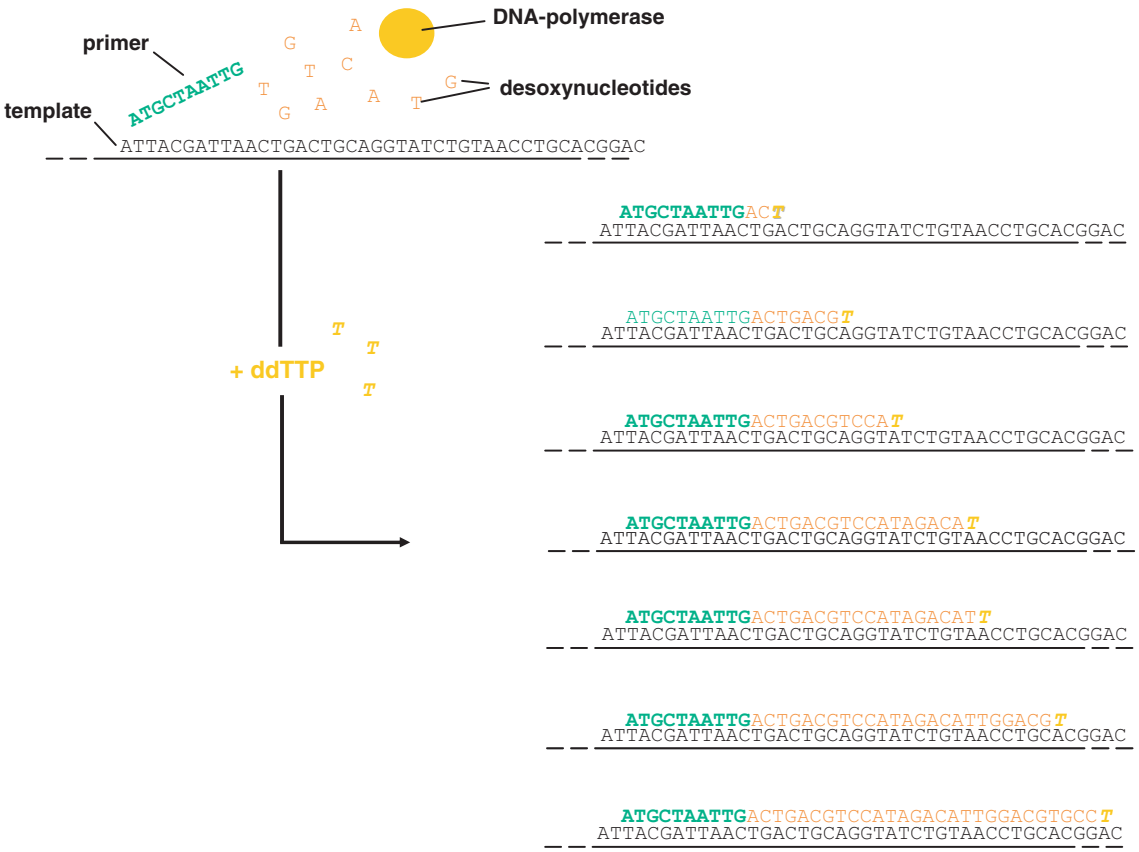

Fig. 1.35 Principle of DNA sequencing using the dideoxy chain termination method. The addition of e.g., dideoxy thymidine triphosphate nucleotides (ddTTP) to the reaction mixture leads to newly synthesized fragments of varying size which end with thymidine. By separation of the products of the four different reactions (using ddATP, ddTTP, ddCTP, ddGTP) in a gel electrophoresis, the sequence can be read from bottom to top of the gel

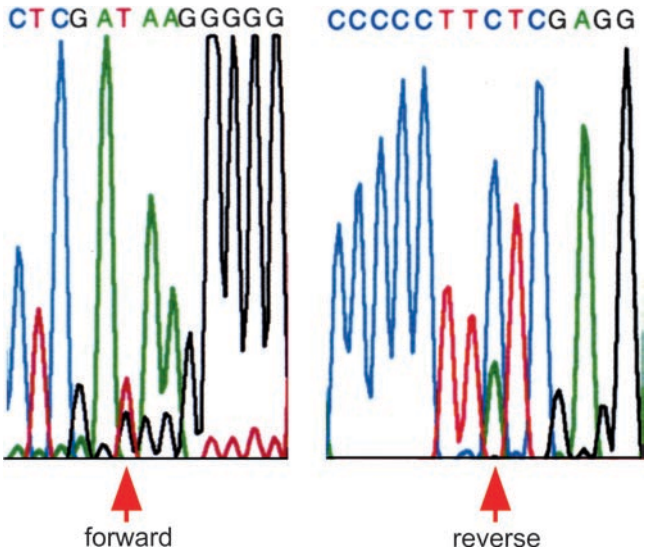

Fig. 1.36 Example of sequencing results using automated non-radioactive cycle sequencing with four different fluorochromes and intensity profiles. Note the double peaks in the forward and reverse sequence (*arrows*) indicating a heterozygous point mutation

sensitivity decreases with increasing size of the probe. The principle of this assay is shown in Fig. 1.37.

DGGE

The DGGE method also relies on the different mobility of wild type and mutant DNA [169]. The target sequences are chosen in order to have a homogenous melting temperature throughout using a software (Win-melt). A so-called GC clamp, a region rich in guanine and cytosine residues 20–60 bp long, is added on to the 5′ end of one primer in order to generate a steep increase of 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, only remaining attached 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

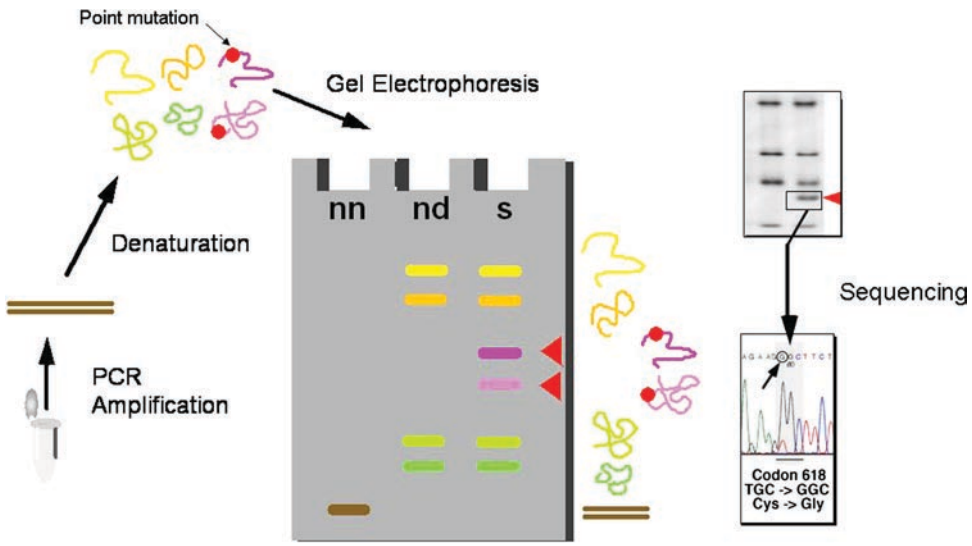

Fig. 1.37 Principles of single strand conformation polymorphism (SSCP) analysis. A point mutation leads to band shifts in the gel electrophoresis (*arrow heads*). *nn* normal DNA non-denatured; *nd* normal denatured; *s* sample DNA

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. 1.38.

DHPLC

This method also relies on different melting properties of mixtures of wild type and mutant DNA. After denaturation and Heteroduplex-formation by gradual re-annealing the PCR fragments are separated over a period of time and over an acetonitrile gradient. The solid phase of the columns has a differential affinity for single- and double-stranded DNA. Heteroduplex DNA has a lower melting temperature and therefore a shorter retention time in the solid phase. If mutated DNA is present in addition to the wild type DNA, the chromatograph pattern will show additional peaks and is distinctive from the control pattern generated by wild type DNA. UV absorption is used to detect the eluted DNA. DHPLC is used in the detection of germline and somatic mutations [170]. Samples with aberrant chromatograph patterns are cycle sequenced.

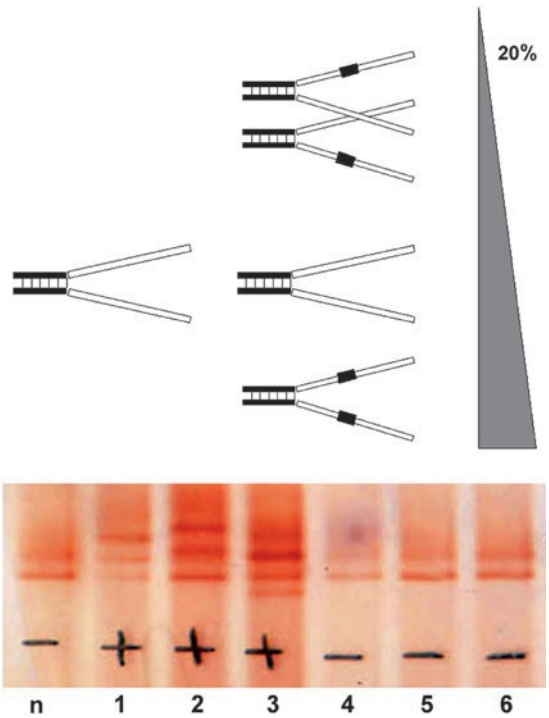

Fig. 1.38 Principles of denaturing gradient gel electrophoresis (DGGE). Separation of PCR products with a point mutation (*right lane*) in a gradient gel leads to additional bands with altered migration when compared to PCR products of wild type DNA (*left lane* and *lower part* of Fig., lanes marked with +). 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)

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, which is not detected by mutation analysis. Germline deletions, however, are an infrequent finding [171, 172]. Mutation analysis is used e.g., for the identification of disease gene carriers of multiple endocrine neoplasia type 1 (MEN1), MEN2 [173, 174] and other familial diseases (Table 1.8) in patients with a suspicious phenotype, and more importantly, in their family members to detect disease-carrier status (Fig. 1.39). 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 (for example, prophylactic thyroidectomy in MEN2B carriers).

Up to 25% of pheochromocytomas and paragangliomas occur as manifestation of a familial syndrome. Germline analyses of the causing genes (*Ret*, *VHL*, *NF1*, *SDHB*, *SDHC* and *SDHD*) therefore, need to be considered [175].

Furthermore, patients with *SDHB*-associated pheochromocytomas/paragangliomas very often have malignant tumors; therefore, germline analysis is important for follow-up of these patients [176].

Mutation analysis in sporadic tumors is – thus far – only used for research purposes; as yet, no somatic mutations with strong clinical impact have been identified [177].

Table 1.8 Familial syndromes with endocrine tumors

Disease	Locus	Gene	Type
Multiple endocrine neoplasia type 1 (MEN1)	11q13	*MENIN*	S
Multiple endocrine neoplasia type 2 (MEN2)	10q11.2	*RET*	O
von Hippel–Lindau (VHL)	3p25.5		S
Neurofibromatose type 1 von Recklinghausen (NF1)	17q11.2	*Neuro-fibromin*	S
Familial paragangliomas	11q23	*SDHD*	S
	1p36.1–35	*SDHB*	
	1q21	*SDHC*	
Mc Cune–Albright	20q13.2	*GNAS1*	O
Carney complex	17q22–24 2p16	*PRKAR1a*	S

SDH succinate dehydrogenase complex; *S* suppressorgene; *O* oncogene

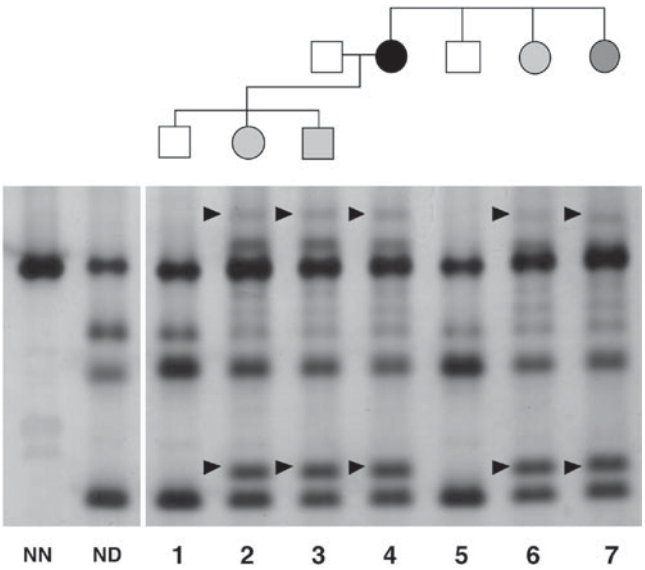

Fig. 1.39 Mutation screening using SSCP analysis of *RET* exon 10 in a MEN2 family. Note that not only the index patient with a medullary thyroid carcinoma (*black circle*) but also two of her children and two sisters exhibit pathological band shifts (*arrow heads*), indicating a disease gene carrier status

1.3.3.5 Loss of Heterozygosity Analysis Using Microsatellite Markers

According to the Knudson hypothesis [178], 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 mutation (leading to an inherited disease as MEN) or somatic (leading to a sporadic tumor). The second mutation is often a large genomic loss of a part of a chromosome or even a whole chromosome. This loss of heterozygosity (LOH) frequently leads to loss of polymorphic markers flanking the tumor-suppressor gene [179]. 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. Since they are usually less than 100 bp long and are flanked by DNA with unique sequences, they can be amplified in vitro using the polymerase chain reaction (Fig. 1.40). 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 in order 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

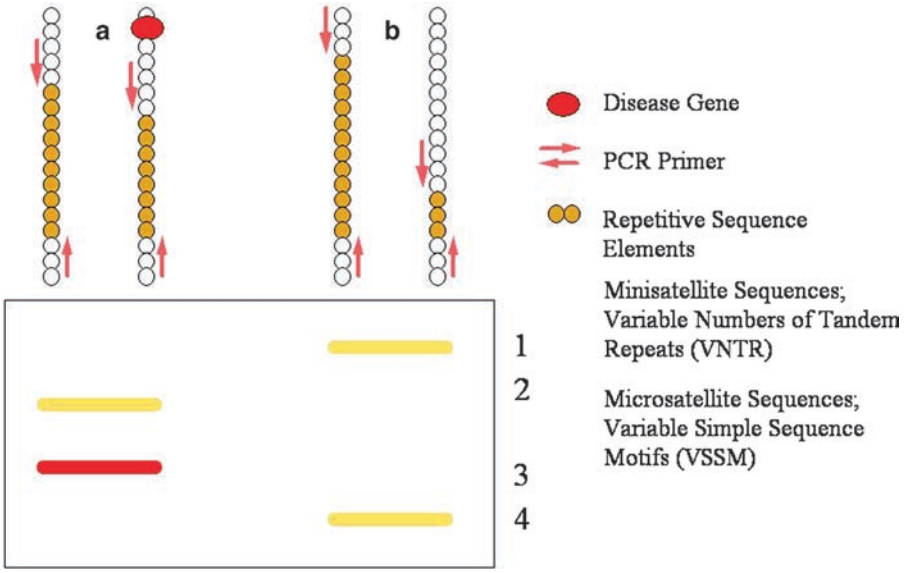

Fig. 1.40 Schematic representation of microsatellite analysis using PCR. Primers flanking a polymorphic region are used to amplify the maternal and paternal alleles of a chromosomal region (note the four different alleles identified in two different individuals). Loss of one allele in tumor tissue is indicative for loss of heterozygosity of a tumor suppressor gene in the vicinity of the polymorphic region

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 be non-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. 1.41). Interpretation is more difficult in tumors with admixture of non-neoplastic tissue as inflammatory cells or vessels. In these 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 [180].

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. While 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 *rare* in endocrine tumors [181–184].

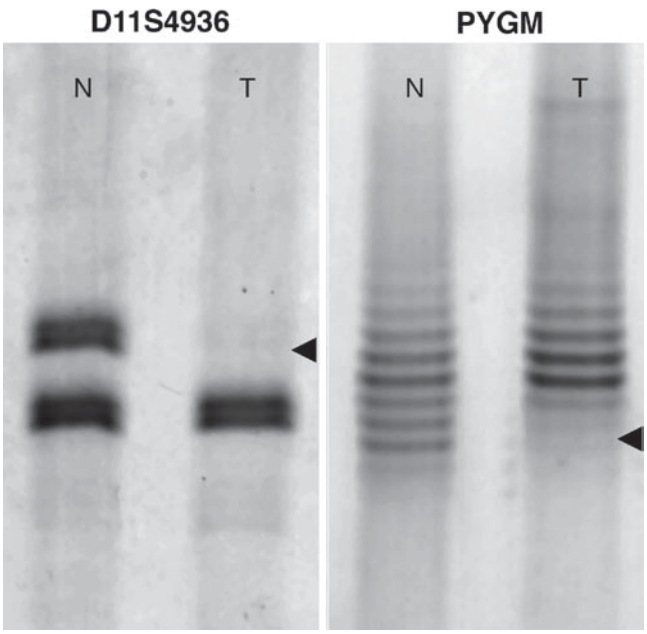

Fig. 1.41 Example of allelic loss of chromosome 11q in an endocrine pancreatic tumor using two different polymorphic markers (D11S4936; PYGM). Note the lost bands (*arrow heads*) in the tumor DNA (T) when compared to the DNA of non-tumorous tissue of the same individual (N)

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 [113, 116]. 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 [167, 185–187] and those results may help to identify tumor-suppressor genes, which are involved in the neoplastic transformation and progression of these neoplasms.

1.3.3.6 Comparative Genomic Hybridization

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 co-hybridized 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. 1.42 and 1.43).

The principal advantages of CGH are: (1) it maps changes in copy number throughout a complex genome onto a normal reference genome so the aberrations can be easily related 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, since 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 (e.g., balanced translocations, inversions etc.)

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 comprising targets spanning >100 kb of genomic sequence such as BACs [188]. More recently, STS-mapped YAC clones were used as targets achieving more than doubled coverage of the chromosomal region of interest. 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 [188]. The high resolution of CGH matrix array compared with chromosome CGH and the opportunities for quantitative aberration definition are apparent in genomic analysis, since the approach of microarray CGH has now been demonstrated in several laboratories [188–190].

Matrix-CGH has been demonstrated [191] on adrenocortical and pancreatic endocrine tumors [192]. Zhao et al. [191] used a microarray-based comparative genomic hybridization (CGH) technique, combined with conventional CGH, to identify 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 since 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 mainly used for research purposes not only in endocrine pathology but also other organ systems. Recent applications of CGH in endocrine neoplasms are listed in Table 1.9.

1.3.3.7 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. 1.44). The highly parallel data acquisition and data

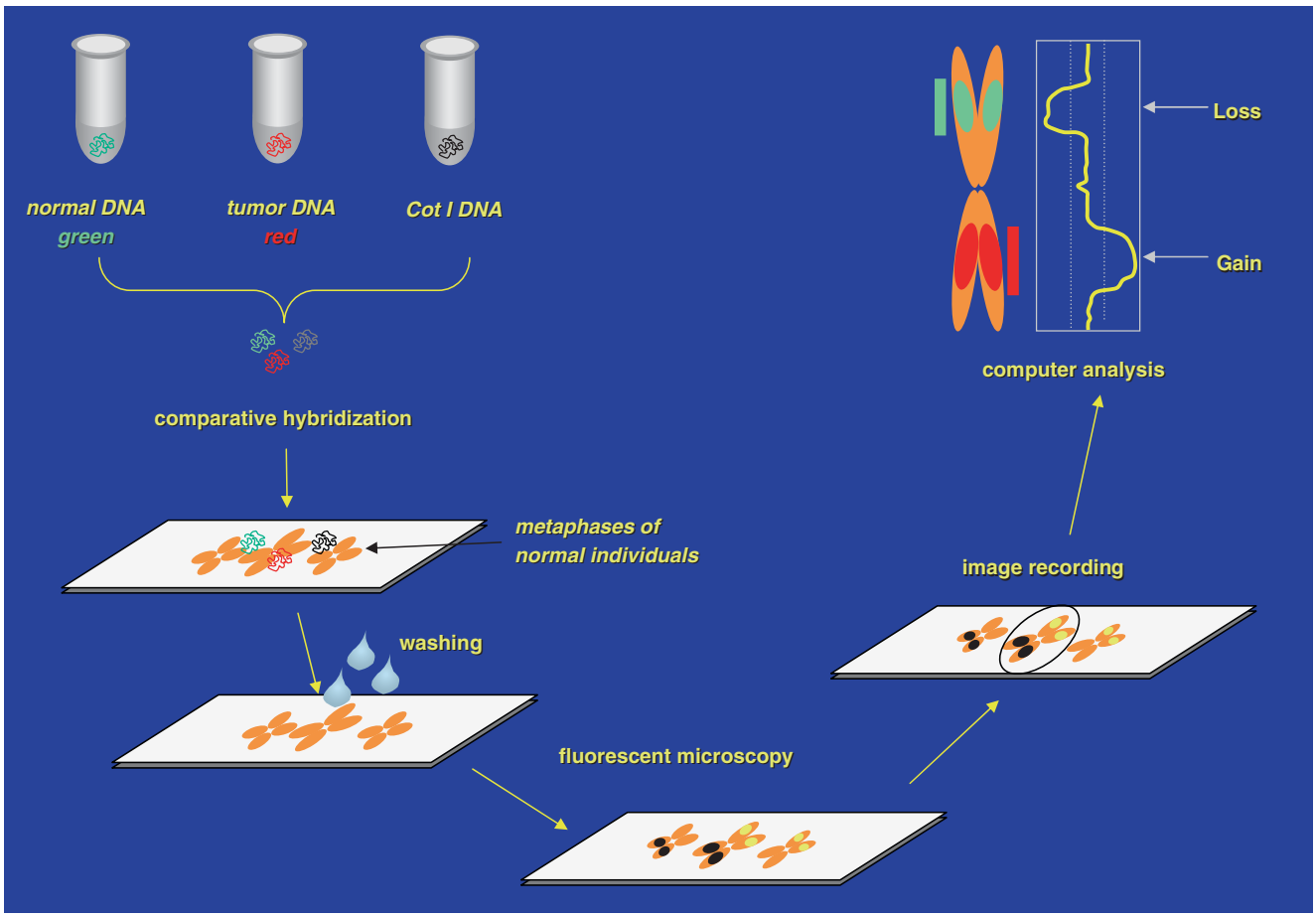

Fig. 1.42 Principles of comparative genomic hybridization (CGH)

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 only reliable and meaningful, *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. Since 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 10^5–10^6 mRNA molecules. Non-amplified RNA from microdissected tissue samples has been used as a radioactive probe for cDNA arrays; however, at least 5,000–50,000 microdissected cells are required for this type of analysis [203, 204].

An array is an orderly arrangement of samples. It provides a medium for matching known and unknown DNA samples based on base-pairing 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 less than 200 µm in diameter and these arrays usually contain thousands of spots. Microarrays require specialized robotics and imaging equipment.

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

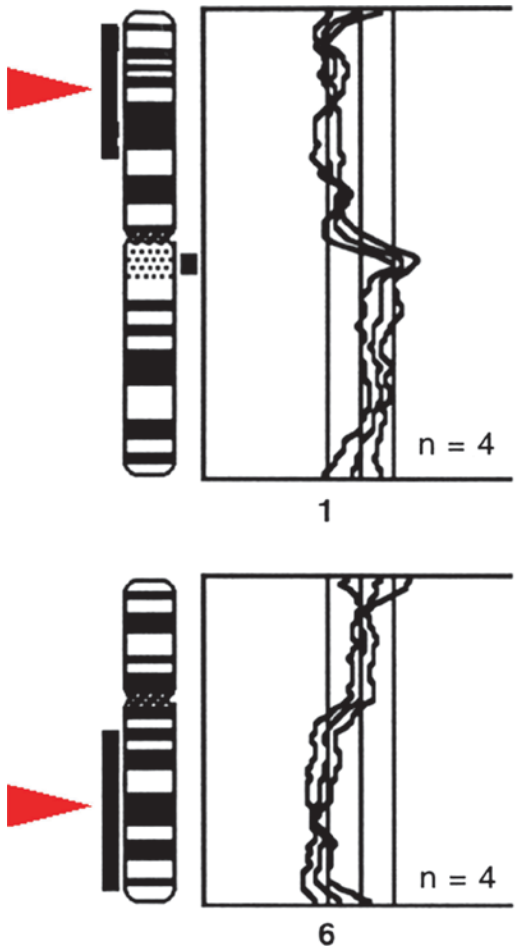

Fig. 1.43 Example of a CGH result in an endocrine pancreatic tumor. The *arrow heads* mark losses of DNA on the short (p) arm of chromosome 1 and on the long (q) arm of chromosome 6

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 [205]. Recent applications of expression arrays in the research of endocrine neoplasms are listed in Table 1.10.

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 further characterized 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 in situ hybridization) or genotypic (fluorescence in situ hybridization) alterations, holding strong potential for translational research. Tissue microarrays consisting of 0.6-mm biopsies of paraffinembedded tissues (Fig. 1.45) are well validated and have been used for various clinicopathological studies [209].

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 efficiently analyze tissue expression of genes identified by DNA microarray

Table 1.9 Selection of chromosomal changes detected by CGH in endocrine tumors

Involved chromosomes/chromosomal regions	Related to	References
Gain of 1q and loss of 9q21.3–q32	Prognosis in papillary thyroid carcinoma	[193]
Chromosomes 19q, 19p, 13q and 11q	Medullary thyroid carcinomas	[194]
Chromosomes 5 and 8	Anaplastic thyroid carcinoma	[195]
Loss on 16p	Anaplastic thyroid carcinoma	[196]
Gain of chromosomes 7, 5 , 9, 12, 14, 17, 18 and X	Benign and malignant follicular thyroid tumours	[197]
Gains of chromosomes X, 19, 12, 7 and 9 Loss of chromosomes 11, 13 and 10	Sporadic pituitary tumors	[198]
3p25.3–p23 loss	Pancreatic endocrine tumors	[113]
9q34 gain	Pancreatic endocrine tumors	[114, 115]
6q22, 6q23–q24 loss	Pancreatic endocrine tumors	[116]
Gain on 4q, 5q (5q13→5q23), 9p (9p21→9pter), 13q (13q21→13q32), 17q	Nonfunctioning pituitary tumors	[199]
Loss of chromosome 11q	Paragangliomas	[200]
Losses of chromosomes 1p and 3q	Sporadic pheochromocytomas (as early genetic events)	[201]
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	[202]

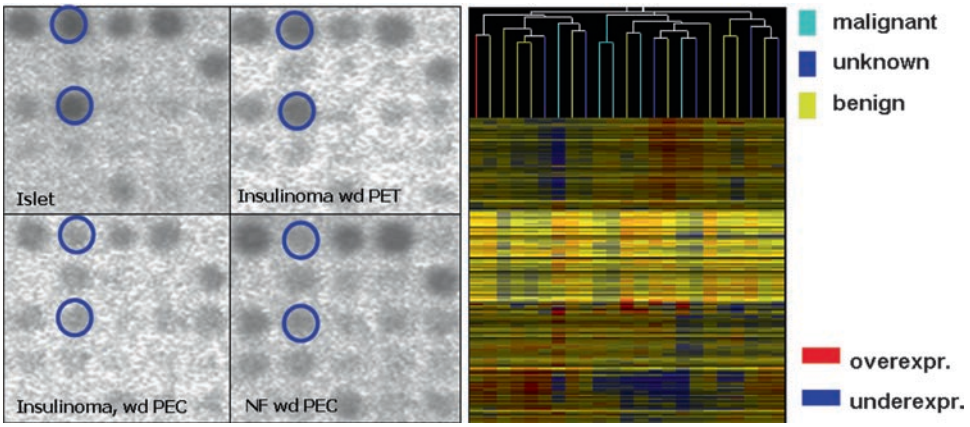

Fig. 1.44 Example of a cDNA expression array experiment using radioactively labeled cDNA: The gene circled in *blue* is expressed in a pool of normal islets and a benign insulinoma, whereas the expression is reduced in malignant insulinomas. Unsupervised cluster analysis of 15,000 genes in 22 insulinomas reveals a clustering of malignant tumors and tumors of unknown behavior

Table 1.10 Recent applications of cDNA microarrays in endocrine pathology

Selection of differentially expressed genes detected by cDNA microarray	Related to	References
MET, LGALS3, KRT19, DPP4, MDK, TIMP1, FN1,CITED1, CHI3L1, ODZ1, N33, SFTPB, and SCEL	Papillary thyroid carcinoma	[206]
Folate receptor gene, ornithine decarboxylase gene, C-mer proto-oncogene tyrosine kinase gene	Pituitary adenomas	[207]
Matrix CGH	Mouse islet carcinomas	[208]

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 intra-assay 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 [43, 46, 117, 210].

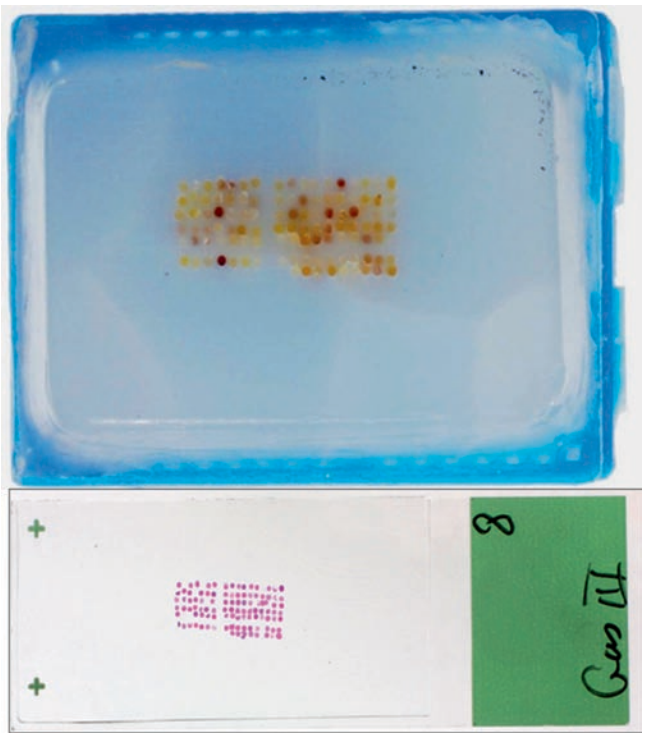

Fig. 1.45 Example of a tissue microarray for pancreatic endocrine tumors (*upper part:* paraffin block; *lower part:* H&E-stained glass slide of tissue section)

1.3.3.8 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

Cells of females carry the 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 [211, 212]. As this process is stable during subsequent cell divisions [213], 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. 1.46).

Different activities of the X-chromosomal *glucose-6-phosphate dehydrogenase (G6PD) isoenzymes* were the first approach to analyze clonality using X-inactivation patterns [214]. However, the low frequency of heterozygosity (2%) for the G6PD isoenzymes made this approach impractical. Methylation-sensitive restriction enzymes, which selectively cleave non-methylated DNA permit to discriminate 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 phosphoribosyl transferase (HPRT) gene* was applied [215]. 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 ~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)* [216] with nearby methylation sensitive restriction endonuclease sites for Hpa II and Hha I made a PCR-based approach possible (Fig. 1.47). These methylation-sensitive enzymes only cut the DNA strand 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 poly-

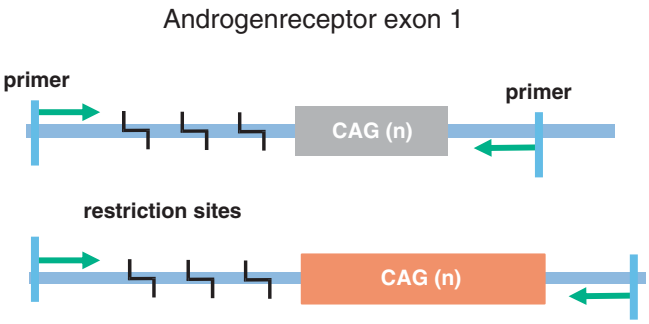

Fig. 1.47 Exon 1 of the androgen receptor gene. A highly polymorphic short tandem repeat sequence (CAGn) close to the methylation sensitive restriction sites *Hha* I or *Hpa* II allows for a specific amplification of the methylated (inactive) allele using restriction enzyme digestion prior to PCR

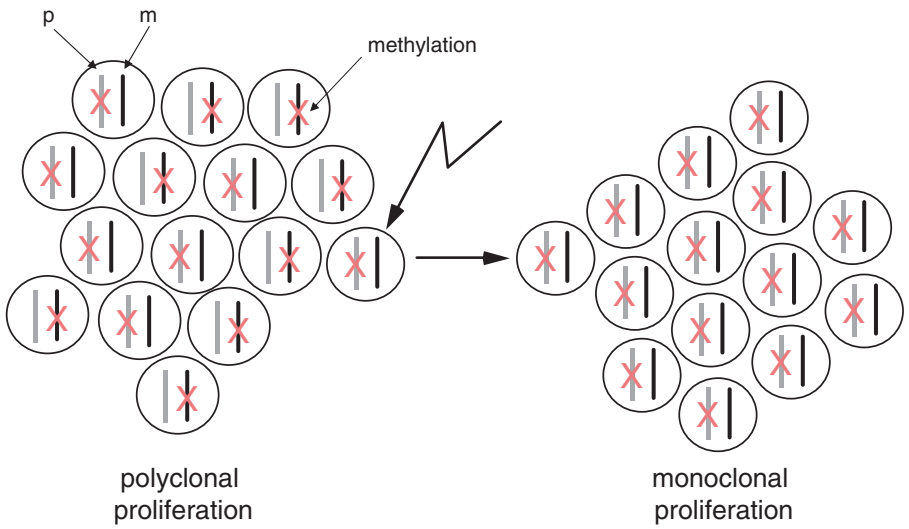

Fig. 1.46 Schematic representation of X-chromosomal inactivation and clonality in a neoplasm of a female patient. Normal tissue in adult females is a cellular mosaic differing in which of the X-chromosome is methylated (polyclonal proliferation). In contrast, monoclonal proliferations are derived from a single somatic cells and thus show a uniform pattern of X-chromosomal inactivation. *p* paternal allele; *m* maternal allele

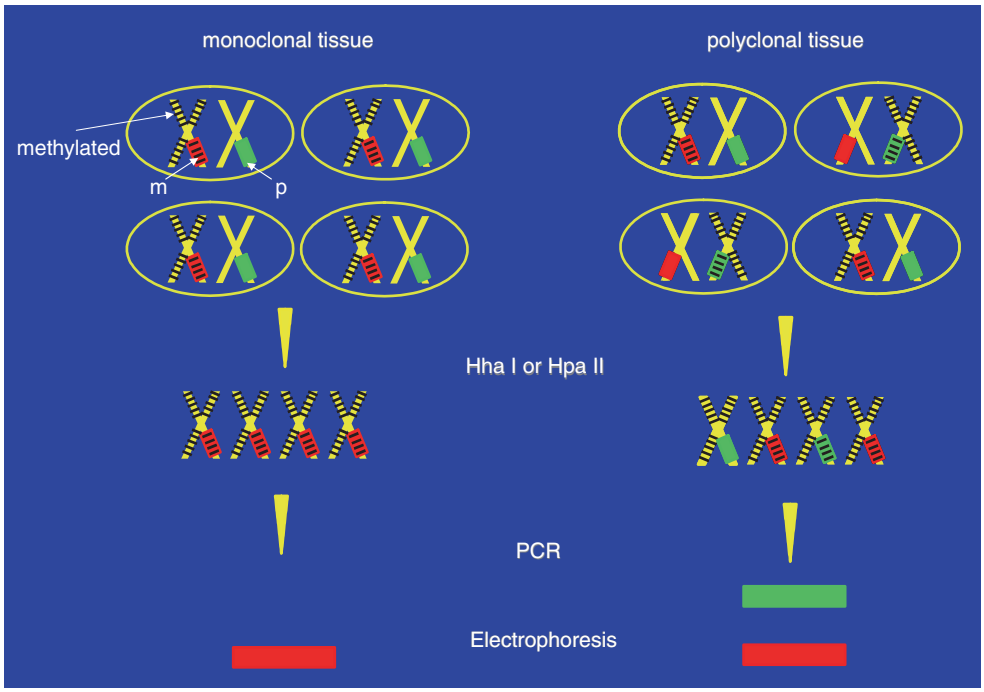

Fig. 1.48 Schematic representation of clonality analysis using the HUMARA assay. The active allele of the X-chromosome is selectively cleaved by restriction analysis prior to PCR amplification of the polymorphic short tandem repeat. Disappearance of one band when compared to normal tissue of the same individual indicates monoclonality. *m* maternal allele; *p* paternal allele

acrylamide gel and visualized by autoradiography or by silver staining (Fig. 1.48). As for LOH analysis, the use of fluorescent primers permits the 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. 1.49).

Methylation sensitive PCR [217] is a different approach, which 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 the mRNA expression [218].

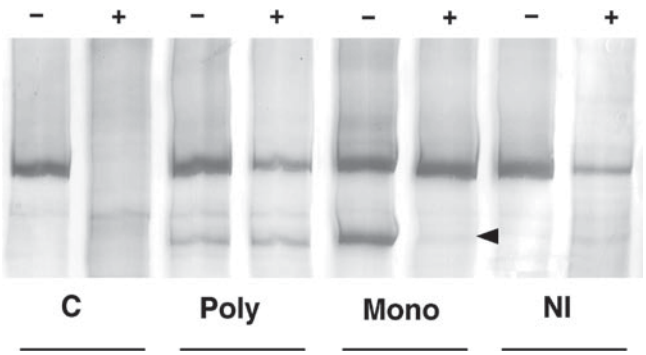

Fig. 1.49 Example of clonality analysis. −: no digestion; +: PCR products after digestion; C: control DNA (male); Poly: polyclonal tissue; Mono: monoclonal tissue; NI: not informative (both alleles have the same length of CAG repeat)

Applications of Clonality Analysis to Endocrine Pathology

Clonality analysis in endocrine tumors has led to intriguing results, as histologically hyperplastic processes [219] can be monoclonal and neoplastic processes can be polyclonal [136]. Therefore, it seems today that the definition of a neoplasm can not solely rely on monoclonality [220–224].

1.4 Future Aspects

It is anticipated that with the now available techniques and the information provided by the completed human genome project, the molecular basis of many endocrine neoplasms will soon be found out. In consequence, our daily routine work as pathologists may look somewhat different in a couple of years. It may include the application of new assays on slides or extracted tissues, of additional immunohistochemi-

cal 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 keep involved in the progress of research by at least ensuring that only tissues of well-characterized tumors and properly monitored patients are included in ongoing studies in order to achieve the goal – to resolve the mystery of endocrine neoplasms.

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This chapter is dedicated to Hubert J. Wolfe, a pioneer in molecular endocrine pathology.

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Chapter 2
Hypothalamus and Neurohypophysis

Kazuhiro Takahashi, Osamu Murakami, and Toraichi Mouri

2.1 A Brief Historical Overview

An English scientist, Geoffrey Harris first seriously urged the idea that the brain controls 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 impedes the pituitary hormone production. However, it was not until discoveries of the hypothalamic hormones by Guillemin and Schally that Harris's theory was proved. Hypothalamic hormones, which are secreted by the hypothalamic neurons and regulate the anterior pituitary hormones (Table 2.1), were mostly discovered by two competitive researchers, Dr. Roger Guillemin and Dr. Andrew Schally [1–3]. Thyrotropin-releasing hormone (TRH), a peptide consisting of three amino acids, was the first-identified hypothalamic hormone [4, 5]. The discovery of the hypothalamic hormones was 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 Dr. Guillemin and Dr. Schally was so competitive that it was called "the Nobel Duel." Both the research groups had aimed at the discovery of corticotropin-releasing hormone (CRH), a hypothalamic hormone which is secreted by the stimuli of "stress" and releases adrenocorticotropin (ACTH) from the anterior pituitary, but without success. Although both Dr. Schally and Dr. Guillemin were endowed with the Nobel Prize in 1977 [1–3], the identification of CRH had to await the isolation of CRH from the ovine brain by Vale et al. [10] and the subsequent molecular cloning of human CRH gene by Shibahara et al. [11].

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Another recent advance in the hypothalamic research has been promoted by the discovery of leptin, a peptide hormone secreted by the adipose tissue [12]. Leptin acts on the brain, in particular, on the hypothalamus and suppresses appetite. Moreover, leptin controls the energy expenditure centrally. Thus, a novel endocrine axis, the adipocyte-hypothalamic axis, has been revealed. 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 the appetite and some of these neuropeptides appear to be under the control of leptin. Furthermore, some gut hormones act on the brain as circulating hormones or via the vagal nerve, and regulate the appetite. For example, ghrelin secreted from the stomach stimulates the appetite [13, 14], whereas peptide YY (PYY) secreted from the intestine suppresses it [15]. 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 concerns in medical care in the twenty-first century. Thus, the hypothalamus appears to play a key role in the pathogenesis of many common diseases, including obesity.

2.2 Physiology and Anatomy of the Hypothalamus

The hypothalamus plays essential roles in the central regulation of hormone secretion in most of endocrine organs, as well as a variety of autonomic functions such as the regulation of appetite, reproduction, temperature, water-electrolyte metabolism, circulation, emotional states, and sleep. The hypothalamus is located at the basal area of brain between the optic chiasm (anteriorly), and the mammillary body and the posterior commissure (posteriorly) (Fig. 2.1). The lateral border is the internal capsule and the basis pedunculi. The dorsal limit of the hypothalamus is the horizontal

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Table 2.1 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

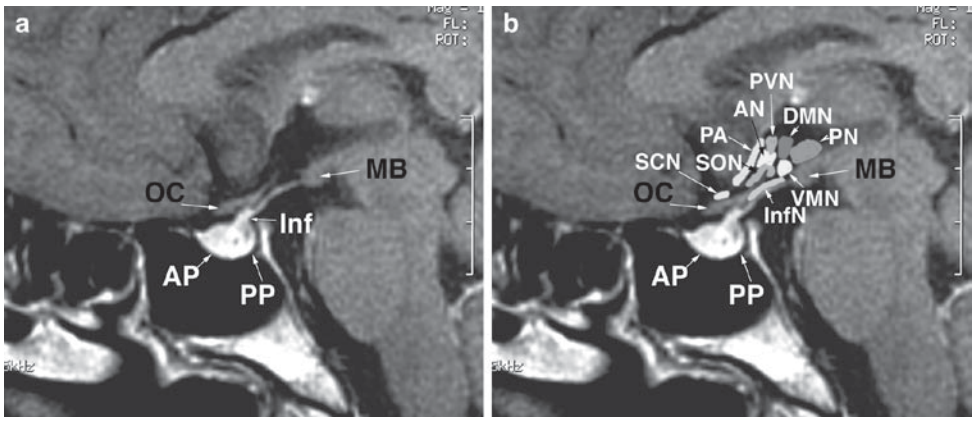

Fig. 2.1 (**a**) Magnetic resonance imaging (MRI) of the brain of a normal subject, and (**b**) the approximate positions of the hypothalamic nuclei. *OC* optic chiasma; *AP* anterior pituitary lobe (adenohypophysis); *PP* posterior pituitary lobe; *Inf* infundibulum; *MB* mammillary body; *SCN* suprachiasmatic 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 and Wilkins 1983.)

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 transport hypothalamic hormones by the axonal transport and release them 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, and regulate the secretion of the respective anterior pituitary hormones. On the other hand, two posterior pituitary hormones, vasopressin and oxytocin, are produced in the magnocellular neurons of paraventricular and supraoptic nuclei, transported to the posterior pituitary lobe via the axonal transport (Fig. 2.2), and released into the systemic circulation. The pituitary (hypophysis) is attached to the infundibulum.

The hypothalamus has mainly two roles in the endocrine regulation: (1) the production and secretion of hypothalamic hormones (Table 2.1), 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

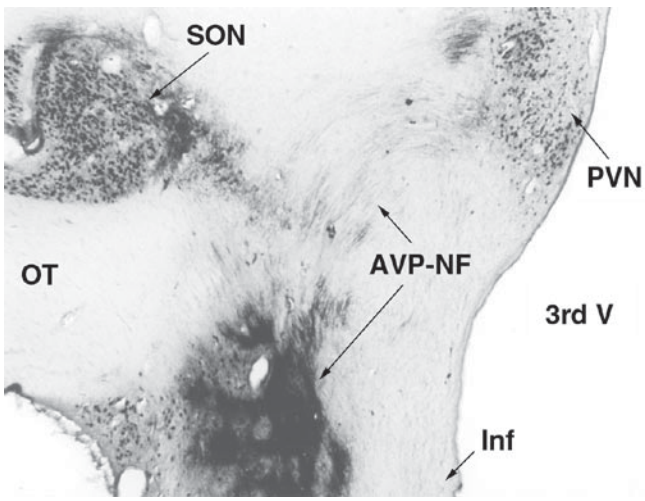

Fig. 2.2 Immunocytochemistry of arginine vasopressin in the human hypothalamus. Vasopressin immunoreactive cell bodies are located in the paraventricular nucleus (PVN) and supraoptic nucleus (SON). Arginine vasopressin-positive nerve fibers (AVP-NF) run from these nuclei toward the infundibulum (Inf) and the pituitary stalk. 3rd *V* the 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.)

synthesized mainly in the parvocellular neurons of the paraventricular nucleus, the periventricular nucleus, and arcuate nucleus of the hypothalamus. For example, CRH is produced in the parvocellular neurons of the paraventricular nucleus (Fig. 2.3). Vasopressin and oxytocin are produced separately in magnocellular neurons of the paraventricular nucleus and supraoptic nucleus of the hypothalamus. Figure 2.2 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 it is co-localized with CRH [16] (Fig. 2.4). Vasopressin has a vasoconstrictor action (mediated by V1 receptor), and an antidiuretic action (mediated by 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 the V3 receptor (or V1b receptor) [17, 18]. Vasopressin and CRH, which are co-localized together in the parvocellular neurons of the paraventricular nucleus, reach the anterior pituitary lobe via the hypophysial portal system and synergistically stimulate the secretion of ACTH.

There are approximately 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 nuclei. There is a complicated neuronal network among these nuclei, and between these nuclei and the extra-hypothalamic brain areas.

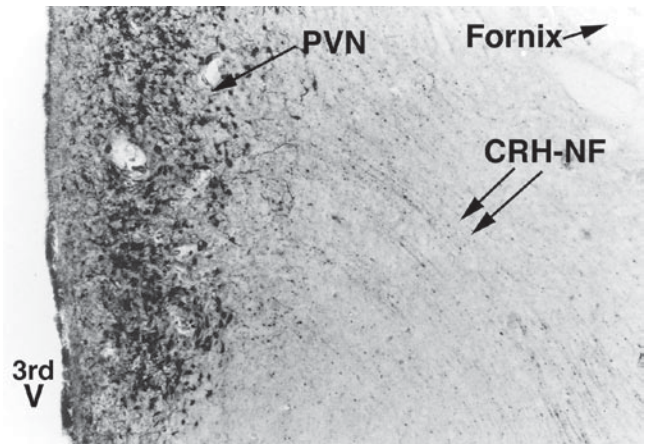

Fig. 2.3 Immunocytochemistry of CRH in the human hypothalamus. CRH-immunoreactive cell bodies are localized in the parvocellular cells of the paraventricular nucleus (PVN). CRH-immunoreactive nerve fibers (CRH-NF) run from these cell bodies laterally and downwards. 3rd V, Third ventricle

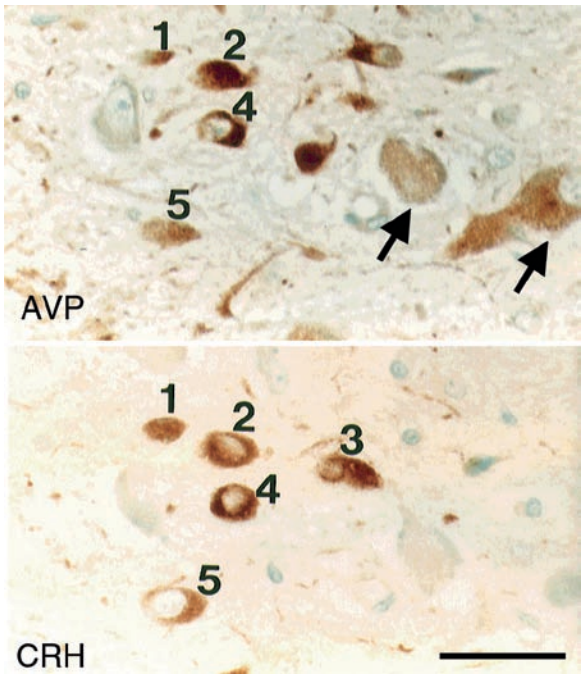

Fig. 2.4 Colocalization of CRH and arginine vasopressin (AVP) in the parvocellular neurons of the paraventricular nucleus of human hypothalamus. CRH is localized in the parvocellular neurons of the paraventricular nucleus and co-localized with vasopressin in these neurons. Vasopressin is also localized in the magnocellular neurons of the paraventricular nucleus and supraoptic nucleus. Vasopressin in the magnocellular neurons is transported to the posterior pituitary lobe by the axonal transport, whereas vasopressin in the parvocellular cells is supposed to be transported to the anterior pituitary lobe with CRH via the hypophyseal portal system. The numbers indicates the identical cell bodies on the serial sections. Arrows indicate AVP-positive magnocellular neurons. Bar = 50 μm

2.3 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 2.1). These hypothalamic hormones are produced in the cell bodies of neurons located in the hypothalamus and transported through nerve fibers by the axonal transport to the median eminence (Fig. 2.3). Hypothalamic hormones are also produced in other brain regions and may act as neurotransmitters or neuromodulators. For example, TRH has been shown to have neurotransmitter actions; it increases locomotor activity, increases body temperature, causes trembling behavior, and decreases food intake, when administered centrally [19].

In addition to these hypothalamic hormones, a number of bioactive substances are involved in the regulation of anterior

pituitary hormone secretion [20]. These include classical neurotransmitters (e.g., noradrenaline, dopamine, acetylcholine), excitatory amino acids (e.g., glutamic acid), various neuropeptides (e.g., kisspeptins [21], neuropeptide Y(NPY), calcitonin gene-related peptide (CGRP) (Fig. 2.5), vasoactive intestinal polypeptide (VIP), pituitary adenylate-cyclase- activating polypeptide (PACAP), (Fig. 2.6), 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.

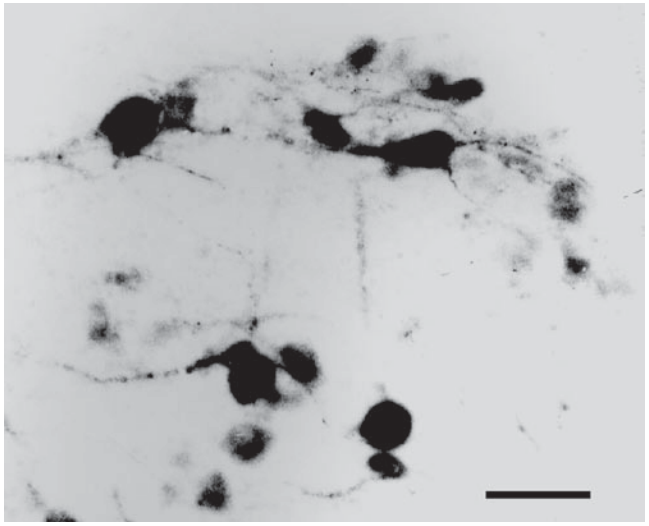

Fig. 2.5 Immunocytochemistry of calcitonin gene-related peptide (CGRP) in the human hypothalamus (supraoptic nucleus). Bar = 50 μm. CGRP is a 37 amino acid peptide which is produced by the alternative processing of the calcitonin gene transcript. CGRP acts as a neurotransmitter or neuromodulator, in 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

2.3.1 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 7 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 have effects on the production and secretion of CRH in the hypothalamus.

In human hypothalamus, CRH is localized in the parvocellular neurons of the paraventricular nucleus (Fig. 2.3) and is co-localized with arginine vasopressin in these neurons (Fig. 2.4) [16]. It is known that a stimulatory effect of CRH on the ACTH secretion is potentiated by vasopressin. In experimental animals, adrenal insufficiency due to adrenalectomy causes increases in the expression of CRH and 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 vasopression 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 hypothalamus but also in extra-hypothalamic 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

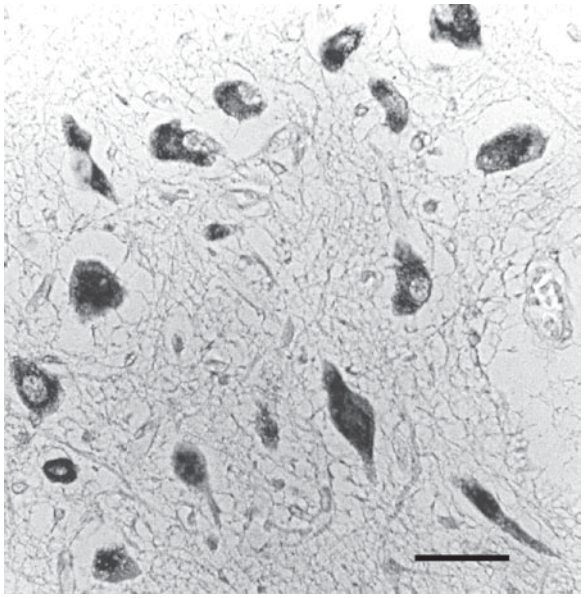

Fig. 2.6 Immunocytochemistry of pituitary adenylate-cyclase activating polypeptide (PACAP) in the human hypothalamus (paraventricular nucleus). Bar = 50 μm. PACAP is a 38- or 27-amino-acid peptide that was isolated from ovine hypothalamus on the basis of the ability to stimulate adenylate cyclase in 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

members in the CRH family peptides: urocortin, stresscopin-related peptide (urocortin II) and stresscopin (urocortin III) [23–25], which are described in the section, *2.3.6 Receptors for hypothalamic hormones and their mutation*.

2.3.2 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 islets cell tumor in a patient with acromegaly [8, 9]. Two major forms were 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 [26, 27], whereas these two peptides are not co-localized 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 the extra-hypothalamic 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 [28]. GH secretagogues act via a specific G protein-coupled receptor: the GH secretagogue receptor [29], not via the GH-RH receptor. Ghrelin is a recently discovered endogenous ligand for GH secretagogue receptor [13]. 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 later section 2.12 “Appetite regulation, obesity, and anorexia nervosa.”

2.3.3 Gonadotropin-Releasing Hormone (Gn-RH) (Luteinizing Hormone-Releasing Hormone LH-RH)

LH-RH is a 10-amino-acid peptide that stimulates secretion of two gonadotropins: luteinizing hormone (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 the rat’s anterior pituitary cells in culture [30].

Gn-RH-expressing neurons migrate during development from their birth place on the medial side of the olfactory placode into the brain [31]. In the adult hypothalamus, Gn-RH-positive cell bodies are most numerous in the ventral and basal hypothalamus [32]. 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. Gn-RH positive nerve fibers project to the posteror pituitary, but the physiological roles of Gn-RH transported to the neurohypophysis remain to be determined. In addition, there are extra-hypothalamic 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 the pulsatile release of Gn-RH is essential for the onset of puberty [33]. Gn-RH secretion and Gn-RH mRNA expression are influenced by estrogen, progesterone, and some neurotransmitters, such as GABA, NPY, opioids, glutamate, and noradrenaline [31, 33]. Estrogen and progesterone increase Gn-RH mRNA in the hypothalamus of rats. GABA reduces Gn-RH mRNA expression levels and its release.

Recently, kissspeptins have been identified to be novel key regulators for Gn-RH [21, 22]. The kisspeptins are the peptide products of the KiSS-1 gene and the endogenous agonists for the GPR54 receptor. Although KiSS-1 was initially discovered as a metastasis suppressor gene, the kisspeptin/GPR54 system has been shown to be a key regulator of the reproductive system. Disrupted GPR54 signaling causes hypogonadotrophic hypogonadism [34]. An activating mutation in the GPR-54 gene resulted in central precocious

puberty [35]. Central or peripheral administration of kisspeptin potently stimulates the hypothalamic-pituitary-gonadal axis, and increases circulating gonadotrophin concentrations in a number of animal models. These effects appear likely to be mediated via the stimulation of the hypothalamic gonadotrophin-releasing hormone system, although kisspeptins may have direct effects on the anterior pituitary gland. Moreover, hypothalamic KiSS-1 gene expression is regulated by circulating sex steroids.

2.3.4 Thyrotropin-Releasing Hormone (TRH)

TRH is a first-discovered hypothalamic hormone consisting of three amino acids (pGlu-His-Pro-NH_2) [4, 5]. TRH is the shortest peptide among the 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 the hypothalamus but also in the other regions of the brain, and may act as a neurotransmitter or neuromodulator. TRH increases locomotor activity, increases body temperature, causes trembling behavior, and decreases food intake, etc. [19].

2.3.5 Hypothalamic Control on the Prolactin Secretion

Prolactin release from the pituitary lactotropes is regulated by a tonic inhibitory control of dopamine derived from the hypothalamus [36]. 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, VIP [37], peptide histidine methionine (PHM), galanin, oxytocin, and prolactin-releasing peptide (PrRP) [38]. 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 is abundant in the gastrointestinal tract and the brain. The VIP precursor generates another bioactive peptide called PHM (peptide histidine isoleucine (PHI) in porcine and rat) by post-translational proteolytic processing [39]. PHM has a structural similarity to VIP and similar biological actions such as vasodilator action and prolactin-releasing activity.

2.3.6 Receptors for the Hypothalamic Hormones and Their Mutations

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 which is predominantly expressed in the pituitary.

CRH receptors consist of two subtypes: CRH receptor type 1 and 2. CRH receptor type 1 is further divided into CRH receptor type 1a, 1b, 1c, 1d, 1e, 1f, 1g, and 1h. CRH receptor type 2 is 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. Particularly, CRH receptor type 1 is expressed in the anterior pituitary lobe and mediates the CRH effect on ACTH release. CRH receptor type 2 is 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 receptor 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 receptor 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 [23–25].

Some mutations in the genes coding these receptors for hypothalamic hormones result in hypopituitary function. There have been several case reports on hypogonadotropic hypogonadism [40, 41], short statue (dwarfism) [42–44], or central hypothyroidism [45], which were caused by the mutations in the Gn-RH receptor, the GH-RH receptor or the TRH receptor, respectively (Table 2.2).

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

Table 2.2 Mutations in the receptors for hypothalamic hormones.

Hypothalamic hormones	Symptoms & signs	Refs
Gn-RH	Hypognadotropic hypogonadism	[40, 41]
GH-RH	Short statue (dwarfism)	[42–44]
TRH	Central hypothyroidism	[45]

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 proteins; 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 [46, 47].

The 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. The McCune Albright syndrome is caused by the mutations in the gene encoding $G_{s\alpha}$ protein, that lead to constitutive activation of $G_{s\alpha}$ and increased cAMP formation [48, 49]. 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 and 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 [48].

2.3.7 Hypothalamic Hormone-Secreting Tumors

Hypothalamic hormone-secreting tumors arise mostly from the peripheral tissues. There are a limited number 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 [40]. There are case reports on intrasellar or hypothalamic gangliocytoma secreting CRH or GH-RH [50, 51]. 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 [52].

2.3.7.1 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 [53, 54]. 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 [54]. Mechanisms underlying this association remain to be determined. One possibility is that the pressure applied by the tumor directly to the hypothalamic centers causes 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 of the presence of Gn-RH in the hypothalamic hamartomas obtained from patients with precocious puberty [55, 56].

2.3.7.2 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 [52].

GH-RH was originally isolated from the tumor tissue of a pancreatic islet cell tumor in a patient with acromegaly [7, 8], and 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 [57]. Although the tumor tissues contain GH-RH, most of the patients with these tumors are free of acromegaly, a symptom which is due to the GH hypersecretion. In only a small number of patients with these tumors, acromegaly occurred clinically [58], possibly because the hypersecretion of GH-RH from the tumor was large enough to cause hypersecretion of GH from the pituitary. 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 [58].

2.3.7.3 CRH-Secreting Tumors

There is one case report of Cushing's disease associated with an intrasellar gangliocytoma producing CRH [51]. Most CRH-secreting tumors, however, arise from the peripheral tissues. Ectopic CRH secretion is usually accompanied by ectopic ACTH secretion. In other words, CRH is expressed

in the tumor tissues of 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 [59] and thyroid medullary carcinoma [60].

It is well-known that pheochromocytomas express a variety of neuropeptides and vasoactive peptides, such as NPY, VIP, GH-RH, somatostatin, CGRP etc. Pheochromocytomas also express ACTH and CRH [61]; 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 that are therefore free from Cushing syndrome. A very limited number of patients with pheochromocytomas show the hormone excess syndromes, such as Cushing syndrome [62].

2.3.7.4 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 [63]. 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.

2.4 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 [64, 65]. 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 to 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 gene 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 days 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 expression levels of vasopressin and oxytocin in the hypothalamus were half those of wild 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 [66, 67]. 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 show 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 the 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 [68]. In homozygous $Otp^{-/-}$ mice, paraventricular, supraoptic and anterior periventricular nuclei were absent, whereas arcuate nucleus was impaired, but present. $Otp^{-/-}$ mice failed 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.

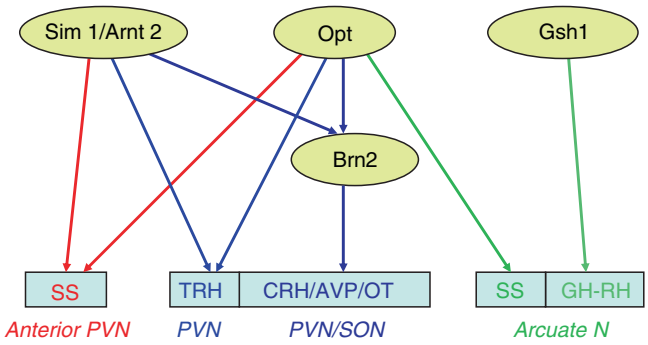

Fig. 2.7 Relationship between transcriptional factors and hypothalamic hormones in the development of hypothalamus. *SS* somatostatin; *TRH* thyrotropin-releasing hormone; *CRH* corticotropin-releasing hormone; *AVP* arginine vasopressin; *OT* oxytocin; *GH-RH* growth hormone-releasing hormone; *PVN* paraventricular nucleus; *SON* supraoptic nucleus

Gsh-1 is a homeobox gene that is essential for GH-RH gene expression in the arcuate nucleus [69]. Gsh-1$^{-/-}$ mice exhibit extreme dwarfism, sexual infantilism, and significant perinatal mortality, with small-sized and hypocellular pituitary.

Figure 2.7 shows summary of the relationship between these transcriptional factors and hypothalamic hormones. On the other hand, there have been no reports on human cases of abnormal hypothalamo-pituitary functions due to mutations of these transcriptional factors.

2.5 Neurohypophysis

The human neurohypophysis is described as consisting of three parts: (1) the median eminence of the hypothalamic tubercinereum; (2) the infundibular stem, which constitutes the pituitary or hypophyseal stalk, together with the portion of adenohypophysis surrounding it (part tuberalis); and (3) the pars nervosa (posterior neural lobe) or infundibular process [70].

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 circulation (Fig. 2.2). 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 anti-diuretic hormone (ADH) following its main actions on the kidney. In humans, rats and mice, vasopressin has an arginine residue at the position 8, and is therefore called arginine vasopressin, while lysine vasopressin found in pigs has a lysine residue at the position 8. The schematic structures of precursors of vasopressin (arginine vasopressin) and oxytocin are shown in Fig. 2.8. 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, or decreases in circulating blood volume, such as hemorrhagic shock, stimulate the release of vasopressin from the neurohypophysis and elevate plasma levels of vasopressin. 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 beta-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 (anti-diuretic action), and V3 pituitary (or V1b) (stimulation of ACTH release) subtypes [17, 18, 71], as described in the previous section, *2.2 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. Furthermore, oxytocin has uterus-contracting actions. These actions are mediated by the oxytocin-specific G-protein coupled receptor. Physiological significance of oxytocin in men and non-pregnant women remains to be determined.

Recent studies have shown that mice lacking oxytocin or oxytocin receptor are both viable and fertile [72, 73]. Oxytocin-deficient female mice, however, had the inability to nurse in spite of normal maternal behavior, and therefore offspring die shortly after birth [72]. Moreover, female mice lacking oxytocin receptor exhibited normal parturition, but demonstrated defects in lactation and maternal nurturing [73]. Adult male mice lacking oxytocin receptor showed deficits in social discrimination and elevated aggressive behavior. Oxytocin may therefore have a neurotransmitter role in the several aspects of social behavior. The defect in oxytocin or oxytocin receptor may be related to elevated

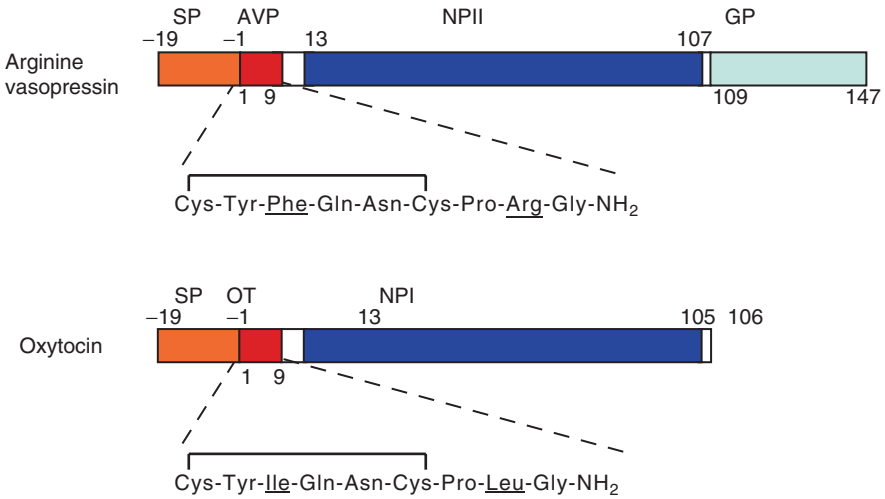

Fig. 2.8 Schematic representation of the precursors of arginine vasopressin (AVP, shown in *red*) and oxytocin (OT, *red*), and the amino acid sequence of AVP and OT. Amino acids different between AVP and OT are underlined. SP, signal peptide (*orange*); NPI, neurophysin I (*blue*); NPII, neurophysin II (*blue*); GP, glycoprotein (*sky blue*)

aggressive behavior in certain psychiatric disorders. Furthermore, there have been reports which show that oxytocin is involved in endocrine and neuroendocrine regulation through receptor mediated actions exerted on the brain, heart, vasculature, and kidneys [74–77]. For example, oxytocin receptor is expressed in the heart, and oxytocin has negative and chronotropic effects on cardiac atrium.

2.6 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. 2.9), etc. Some rare tumors that secrete hypothalamic hormones and much more rare cases of hypothalamic hormone receptor mutations are described in the previous sections. Inflammatory diseases include sarcoidosis, histiocytosis, infundibuloneurohypophysitis etc. 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 water-electrolyte metabolism (diabetes insipidus, hypernatremia or hyponatremia), hypofunction of anterior pituitary, psychic disturbance, abnormalities in appetite (hyperphagia and obesity, or anorexia), emaciation, thermodysregulation, sleep disorders (e.g., narcolepsy), and sphincter disturbances. 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 pituitary 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,

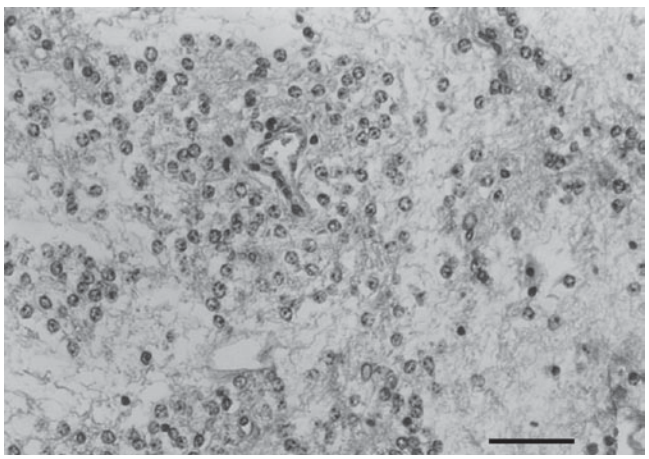

Fig. 2.9 Astrocytoma in the hypothalamus (H & E). Bar = 50 μm (Courtesy of Dr. H. Ikeda, Department of Neurosurgery, Southern Tohoku General Hospital, Koriyama, Japan.)

diabetes insipidus almost always occurs. If it is cut at the lower level, the incidence is less.

2.7 Diseases Due to the Dysfunction in Vasopressin Secretion

2.7.1 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).

2.7.1.1 Central Diabetes Insipidus

Central diabetes insipidus is classified into three categories, familial, secondary, and idiopathic, according to the causes (Table 2.3). Familial diabetes insipidus is a very rare disorder and is characterized by autosomal dominant inheritance. Secondary diabetes insipidus is caused by tumors, infection, inflammatory diseases, infiltrative processes and trauma that damage the hypothalamic-neurohypophysial system. Tumors and inflammatory diseases in the hypothalamus and neurohypophysis are described in the following sections.

Table 2.3 Classification of central (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, lymphoma etc.)
Anterior pituitary tumors (mostly, postoperative)
Infection (tuberculosis, AIDS-associated infection etc.)
Granulomatous diseases (sarcoidosis, Langerhans'-cell histiocytosis, non-Langerhans'-cell histiocytosis, etc.)
Lymphocytic adenohypophysitis
Lymphocytic infundibuloneurohypophysitis
Guillain-Barré syndrome
Aneurysms
Infiltrative processes (hemochromatosis, amyloidosis)
Trauma
Idiopathic diabetes insipidus (Lymphocytic infundibuloneurohypophysitis?)

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 the two alleles of the vasopressin-neurophysin II gene in families with familial diabetes insipidus [78, 79]. 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 [78]; and Val to Gly at amino acid position 17 in the neurophysin II moiety [79]). Neurophysins bind to 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 peptide in these patients [80–82]. 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 many mechanisms.

The mutant arginine vasopressin-neurophysin II complex may accumulate slowly in the magnocellular neurons and lead to the death of these neurons [83]. Autopsy studies of patients with autosomal dominant diabetes insipidus show a markedly subnormal number of magnocellular neurons and associated moderate gliosis [84, 85].

Wolfram Syndrome

Wolfram syndrome is an autosomal recessive neurodegenerative disorder associated with juvenile-onset non-immune insulin-dependent diabetes mellitus, progressive optic atrophy, sensorineural deafness, and diabetes insipidus, also known as DIDMOAD (Diabetes Insipidus, Diabetes Mellitus, Optic Atrophy, and Deafness) [86]. 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 [86]. Central diabetes insipidus occurred in about 73% of the patients [87]. A Wolfram gene (*WFS1*) has been mapped to chromosome 4p16.1 [88]. The *WFS1* encodes an 890 amino acid protein, which is a membrane glycoprotein and localizes in the endoplasmic reticulum [89]. WFS1 is widely expressed in the brain, including hippocampus, amygdaloid, and hypothalamus, consistent with a variety of neurological manifestations and central diabetes insipidus. Although the relationship between the WFS1 deficiency and the hypofunction of hypothalamic vasopressin neurons is not fully clarified, recent studies have shown the relationship between the WFS1 deficiency and pancreatic beta-cell loss [89, 90]. In pancreatic islets of wfs1-deficient mice, WFS1-deficiency increased endoplasmic reticulum stress and caused pancreatic beta-cell loss through impaired cell cycle progression and increased apoptosis [90].

Idiopathic Diabetes Insipidus, Lymphocytic Infundibuloneurohypophysitis, and Lymphocytic Adenohypophysitis

Idiopathic diabetes insipidus comprises approximately 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. [91]. On the other hand, lymphocytic adenohypophysitis is a rare inflammatory disease which primarily affects adenohypophysis and is probably caused by autoimmunity (Figs. 2.10 and 2.11). Approximately 19% of the patients with lymphocytic adenohypophysitis, however, have diabetes insipidus [92]. Lymphocytic infundibuloneurohypophysitis and lymphocytic adenohypophysitis are described in details in the later section 2.9 "Inflammation in the hypothalamus and neurohypophysis."

2.7.1.2 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

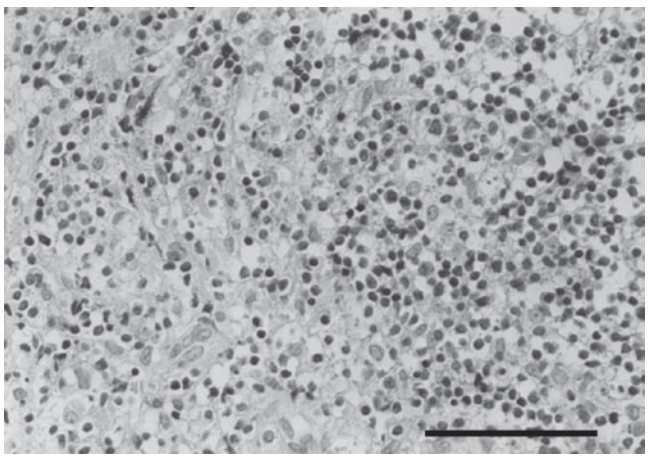

Fig. 2.11 Lymphocytic hypophysitis (H & E). Bar = 50 μm (Courtesy of Dr. H. Ikeda, Department of Neurosurgery, Southern Tohoku General Hospital, Koriyama, Japan.)

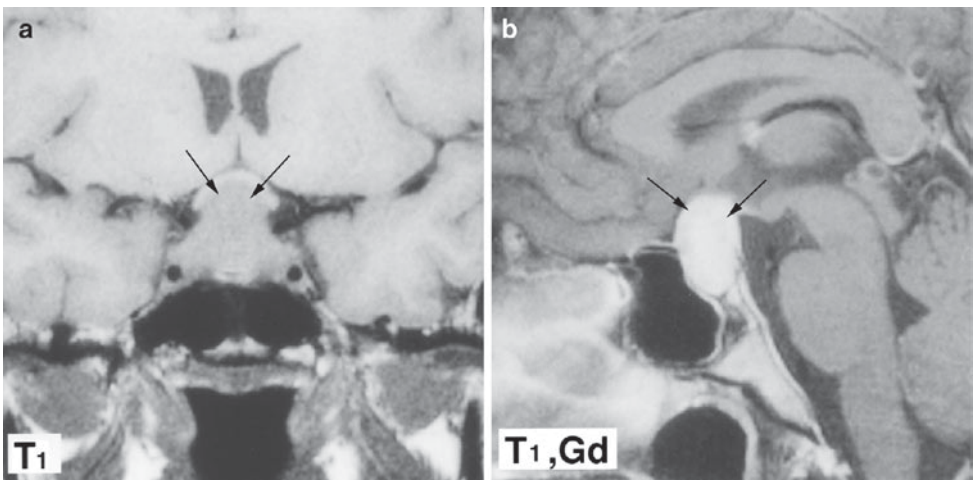

Fig. 2.10 MRI in a patients with lymphocytic hypophysitis. *Arrows* indicate pituitary gland with lymphocytic hypophysitis. (**a**) Coronal T1-weighted MRI scan. (**b**) Sagittal T1 weighted MRI scan after gadolinium enhancement (Reproduced from Takahashi K, Murakami O, Satoh F, Mouri T. The hypothalamus and neurohypophysis. In: Stefaneau L, Sasano H, Kovacs K, eds. Molecular and Cellular Endocrine Pathology. London: Arnold, 2000:45-74, with permission.)

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 patient of congenital nephrogenic diabetes insipidus who had 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 [93].

The activation of the V2 receptor on renal collecting tubules stimulates adenylate cyclase via the stimulatory G protein (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 recessive inheritance is caused by compound heterozygote or homozygosity for mutations in the AQP2 gene [94, 95].

Table 2.4 The diseases most often associated with SIADH.

1. Vasopressin-secreting malignant tumors
2. Non-malignant pulmonary diseases
Asthma, pneumonia, pulmonary tuberculosis, chronic obstructive pulmonary disease
3. Disorders of the central nervous system;
Meningitis, encephalitis, brain abscess, AIDS
Trauma, aneurysm
Multiple sclerosis, Guillain-Barré syndrome, acute intermittent porphyria
Bleeding in the brain (e.g., subdural hematoma, subarachnoid hemorrhage)
Brain tumors (non-vasopressin secreting)
4. Drugs;
Chlorpropamide, tricyclic antidepressants, carbamazepin, selective serotonin-reuptake inhibitor
Clofibrate, nicotine
Vincristine, cyclophosphamide
Nonsteroidal anti-inflammatory drugs, etc.

2.7.2 SIADH (Syndrome of Inappropriate ADH Secretion)

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 vasopressin in spite of a subnormal plasma osmolarity. The diseases most often associated with SIADH are shown in Table 2.4. The most common causes for SIADH are malignant diseases, especially small cell or oat cell carcinomas of the lung [96], which produce and secrete vasopression. The other malignant tumors associated with SIADH include carcinomas of the pancreas, duodenum, bladder and prostate, thymomas, lymphomas, and Ewing's sarcomas. Non-malignant 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 Table 2.4 may directly or indirectly stimulate the hypothalamo-neurohypophysis to secrete vasopressin. Several drugs to stimulate the vasopressin release are also known.

2.7.3 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 [97]. 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 which does not respond to chronic fluid overloading, but which may be corrected by vasopressin administration [98–100]. Thus, the sustained hyperosmolarity in the patients with essential hypernatremia is the result of an elevated osmotic threshold for release of vasopressin. As 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."

2.8 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. 2.9), meningiomas, gangliocytomas, hamartomas, craniopharyngiomas, germinomas, teratomas etc. Hypothalamic hormone-secreting gangliocytomas and hamartomas are described in the earlier section, 2.3.7 "Hypothalamic hormone-secreting tumors." In this section, other tumors and cystic lesions that specifically affect the hypothalamus are discussed. Rathke's cleft cyst arises mainly intrasellarly, and often suprasellarly, and therefore is also described here.

2.8.1 Craniopharyngioma

Craniopharyngioma is a cystic neoplasm derived from the remnants of Rathke's pouch (Fig. 2.12). It originates from the pituitary stalk and is usually suprasellar. Craniopharyngioma can be clinically aggressive with finger-like 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. 2.12). Calcification can be found on X-ray examination in the sellar or suprasellar region.

2.8.2 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. 2.13 and 2.14). 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,

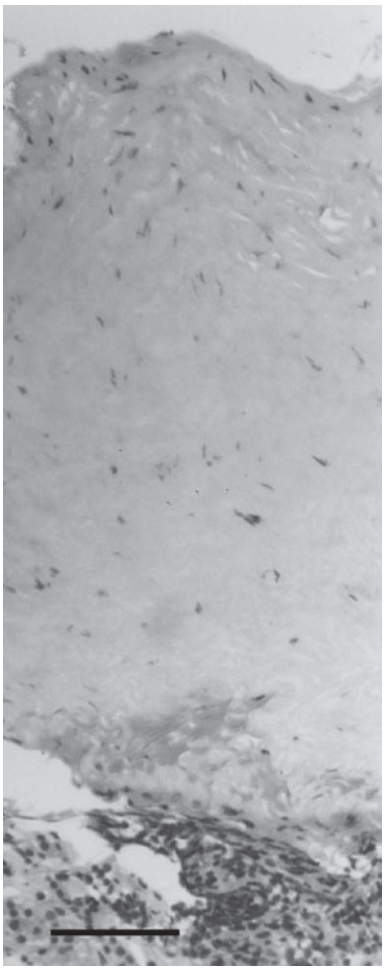

Fig. 2.13 Cyst wall of Rathke's cleft cyst (H & E). Bar = 50 μm (Courtesy of Dr. H. Ikeda, Department of Neurosurgery, Southern Tohoku General Hospital, Koriyama, Japan.)

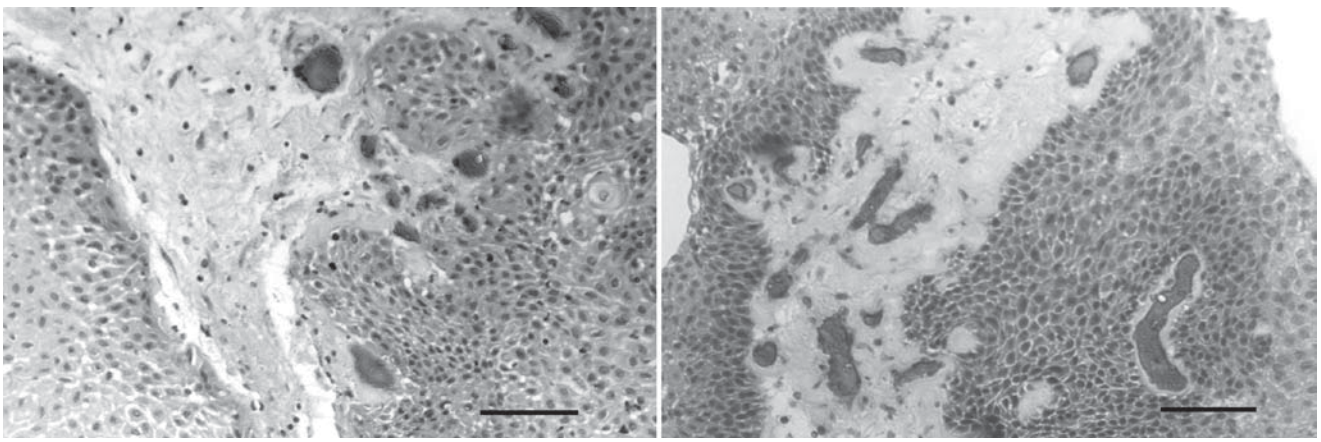

Fig. 2.12 Craniopharyngioma (H & E). Bar = 50 μm (Courtesy of Dr. H. Ikeda, Department of Neurosurgery, Southern Tohoku General Hospital, Koriyama, Japan.)

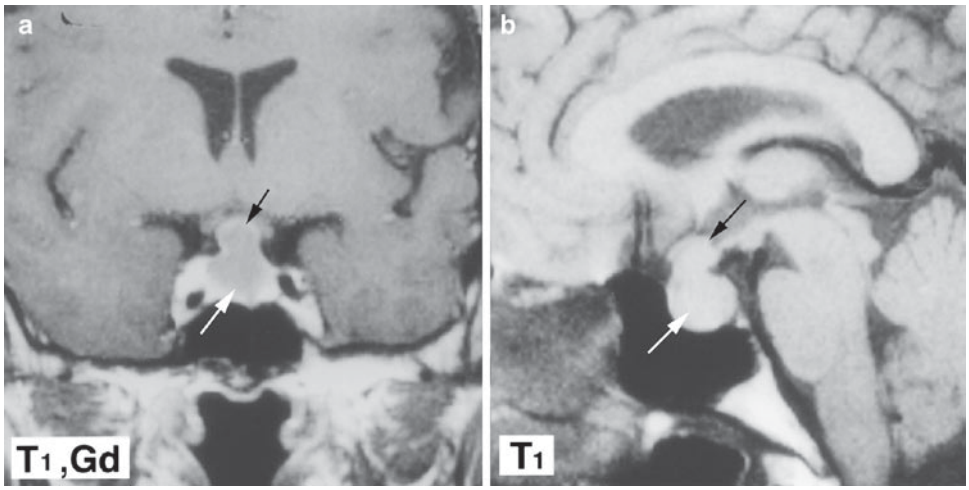

Fig. 2.14 MRI of Rathke's cleft cysts (*arrows*). (**a**) Coronal T1-weighted MRI scan after gadolinium enhancement. (**b**) Sagittal T1-weighted MRI scan. The cyst is located in the suprasellar (shown by *black arrows*) and intrasellar (shown by *white arrows*) regions (Reproduced from Takahashi K, 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.)

hyperprolactinemia, diabetes insipidus, headache, and impairment of visual acuity and visual field defects [101]. A case of Rathke's cleft cyst with SIADH is also reported [102]. Approximately 50% of Rathke's cleft cysts are intrasellar, and the others are suprasellar, or suprasellar and intrasellar [103].

2.8.3 Germ Cell Tumors

Germ cell tumors include germinomas, choriocarcinomas, yolk sac tumors, embryonal carcinomas, teratomas with various degree of differentiation and mixed germ cell tumors. These tumors are presumed to originate from embryonic nests of germ cells, and 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 hypothalamohypophyseal region. Among the germ cell tumors arising in the hypothalamohypophyseal region, germinomas are the most common (suprasellar germinoma) [104]. Approximately 20% of germinomas produce human chorionic gonadotropin [105]. Clinical manifestations of suprasellar germinomas are diabetes insipidus, visual disturbance, and hypopituitarism (growth retardation or hypogonadism).

2.9 Inflammation in the Hypothalamus and Neurohypophysis

Inflammatory diseases (e.g., Langerhans'-cell histiocytosis, Erdheim-Chester disease, sarcoidosis, and lymphocytic infundibuloneurohypophysitis), and infectious diseases (e.g., bacterial, viral, fungal or tuberculous etc.) can affect the hypothalamus and neurohypophysis. While tuberculosis was the most common among the infectious diseases of the endocrine organs, opportunistic infections involving the endocrine organs in patients with acquired immunodeficiency syndrome (AIDS) have been increasingly recognized [106]. A case of central diabetes insipidus due to cytomegalovirus infection of the hypothalamus with AIDS has been reported [107].

Hypophysitis is an inflammatory lesion of the pituitary gland, and usually classified as adenohypophysitis and infundibuloneurohypophysitis depending on the location of the main lesion in the pituitary gland. Adenohypophysitis clinically and radiologically mimics tumors of the sellar region, causing mass effects such as headache and visual impairment. Adenohypophysis primarily affects the anterior pituitary lobe, but may result in hypophyseal and/or hypothalamic dysfunction such as diabetes insipidus from inflammatory destruction of the hypophysis (panhypophysitis) or compression of the residual normal gland by edema. Whereas secondary hypophysitis caused by infection or systemic disease is relatively rare today, primary (idiopathic, or probably autoimmune) hypophysitis is currently the most common form of pituitary inflammation. Primary hypophysitis include three histopathological conditions: lymphocytic hypophysitis, granulomatous hypophysitis, and xanthomatous hypophysitis [108].

2.9.1 Langerhans'-Cell Histiocytosis

Langerhans'-cell histiocytosis is a granulomatous disease that occurs mostly in children between 1 and 4 years of age [109, 110]. 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-Schuller-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, that are known as Birbeck granules [111, 112].

Diabetes insipidus and growth retardation are the prominent endocrine manifestations of Langerhans'-cell histiocytosis [112, 113]. 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.

2.9.2 Non-Langerhans'-Cell Histiocytosis (Erdheim-Chester Disease)

Erdheim-Chester disease is an idiopathic progressive non-Langerhans' cell histiocytosis characterized by xanthogranulomatous infiltration of foamy macrophages [114–116]. This rare disorder (only about 500 cases in the literature) is typically manifested as pain and sclerotic lesions in the long bones, particularly the diaphyses, but extraskeletal involvement is common. The common sites involved are the orbit, the hypothalamo-pituitary-sella area, the retroperitoneal and periaortic tissues, the lungs and the heart. Infiltration to the pituitary stalk sometimes causes diabetes insipidus [115, 116].

2.9.3 Sarcoidosis

The hypothalamus and pituitary is the most commonly affected regions by sarcoidosis although endocrine manifestation is relatively rare in sarcoidosis [117–119]. The central nervous system is involved in about 5% of all cases of sarcoidosis and diabetes insipidus occurs in 33% patients with neurosarcoidosis [120]. In addition to diabetes insipidus, patients with sarcoidosis often exhibit hypothalamic disturbances and anterior pituitary hormone deficiency [117–119]. Histologically, sarcoidosis shows noncaseous granulomatous tissue with multinucleated giant cells of foreign body type.

2.9.4 Lymphocytic Infundibuloneurohypophysitis and Lymphocytic Adenohypophysitis

Lymphocytic infundibuloneurohypophysitis was proposed to be one major cause of what was previously considered to be idiopathic diabetes insipidus by Imura et al. [91]. They studied 17 patients with idiopathic diabetes insipidus. Magnetic resonance imaging (MRI) showed that nine 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 years, 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.

Autoimmune central diabetes insipidus is diagnosed based on the presence of autoantibodies to arginine vasopressin-secreting cells or the coexistence of other autoimmune polyendocrine syndromes. Vasopressin-cell antibodies have been detected with a high incidence (15 out of 22 cases) among patients with complete autoimmune central diabetes insipidus [121]. Pituitary stalk thickening on MRI which suggested lymphocytic infundibuloneurohypophysitis occurred only in patients with positive vasopressin-cell antibodies [121].

Lymphocytic adenohypophysitis is a rare inflammatory disease which primarily affects adenohypophysis and is probably caused by autoimmunity (Figs. 2.10 and 2.11). Approximately 19% of the patients with lymphocytic adenohypophysitis, however, have diabetes insipidus [92, 122]. 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 adenohypophysitis has an acute onset. Clinical

manifestations are headaches, visual symptoms and signs, hypopituitarism and radiological appearance of sellar mass lesion which mimics pituitary adenoma (Fig. 2.10). Lymphoplasmacytic infiltrate is a histological feature of this disease with occasional infiltrate of neutrophils, eosinophils, and macrophages in the anterior pituitary gland (Fig. 2.11). The inflammatory infiltrate was shown in the neurohypophysitis of some patients with lymphocytic adenohypophysitis and diabetes insipidus.

2.9.5 Other Autoimmune Diseases

Systemic autoimmune diseases, such as giant-cell arteritis [123] and Wegener's granulomatosis, affect the hypothalamus, the hypophysis and/or the meninges around the sella turcica. In addition, lymphocytic adenohypophysitis and lymphocytic infundibuloneurohypophysitis are frequently associated with autoimmune diseases, such as systemic lupus erythematosus [124]. Circulating anti-pituitary antibodies were detected at higher frequencies in patients with autoimmune thyroid disorders (Hashimoto's thyroiditis, 13%; Graves' disease, 7.1%) than in control subjects (0.9%), suggesting that autoimmune hypophysitis is likely to be much more common than previously thought [125, 126]. Thirty-six out of 110 subjects with positive circulating anti-pituitary antibodies had mild or severe GH deficiency and one had central diabetes insipidus [125].

2.10 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 neurohypophyseal masses include neoplastic, infiltrative or granulomatous diseases. Primary neoplasms originating from the posterior lobe are extremely rare; the most common are granular cell tumors (choristomas or pituicytomas) [127]. The majority of such tumors remains asymptomatic and is 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 Langerhan's histiocytosis, sarcoidosis, tuberculosis, and syphilis, which can infiltrate the posterior lobe or pituitary stalk to cause diabetes insipidus [128].

2.11 Hypogonadotropic Hypogonadism and Kallmann's Syndrome

Hypogonadotropic hypogonadism is characterized by failed gonadal function secondary to deficient LH/FSH secretion [129]. Hypogonadotropic hypogonadism is caused by dysfunction of gonadotropic cells due to pituitary lesions, such as tumors, inflammatory diseases, and infiltrative processes (e.g., iron deposition by hemochromatosis) [130], or Gn-RH deficiency in the hypothalamo-pituitary axis. Idiopathic hypogonadotropic hypogonadism is a congenital disorder characterized by Gn-RH deficiency, classically presents with delayed or absent puberty and has a prevalence of about 0.025% in males and about 0.01% in females. About 50% of cases of idopathic hypogonadotropic hypogonadism are accompanied with congenital anosmia and constitute Kallmann's syndrome. We describe here hypogonadotropic hypogonadism caused by abnormalities in the hypothalamus.

2.11.1 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 is by the absence of olfactory bulbs and tract. This syndrome is genetically heterogenous and can be transmitted as an X-linked, autosomal dominant or autosomal recessive trait.

Loss-of-function mutations in KAL1 and fibroblast growth factor receptor 1 (FGFR1) gene (FGFR1/KAL2) underlie the X-linked form and an autosomal dominant form of the disease, respectively [131, 132]. KAL1 mutations result in a more severe reproductive phenotype than FGFR1/KAL2 mutations [133]. Fibroblast growth factor 8 (FGF8) is a ligand for FGFR1, and loss-of-function mutations in FGF8 underlie both Kallmann's syndrome and normosmic idiopathic hypogonadotropic hypogonadism [134]. Furthermore, loss-of-function mutations in genes encoding the G protein-coupled prokineticin receptor-2 (PROKR2) and one of its ligands, prokineticin-2 (PROK2) have been shown to underline both Kallmann's syndrome and normosmic idiopathic hypogonadotropic hypogonadism [135, 136].

The KAL1 gene was located in the critical region on Xp22.3, and encodes anosmin-1, a locally restricted glycoprotein of embryonic extracellular matrices, which has significant similarities with proteins involved in neural cell adhesion and axonal path-finding, and may have a specific role in neuronal migration. There is a functional interaction between anosmin-1 and the FGFR1-FGF2-heparan sulfate complex, leading to amplified responses in the FGFR1 signaling pathway during the nervous system development [137]. The PROK2 signaling system regulates diverse biological processes, including olfactory bulb morphogenesis, and reproduction, through multiple intracellular signaling pathways, including calcium mobilization.

2.11.2 Other Hypogonadotropic Hypogonadism Related to the Hypothalamus

In addition to KAL1, FGFR1/KAL2, FGF8, PROKR2, and PROK2, loss-of-function mutations in genes of Gn-RH Receptor (GNRHR)[40, 41], Nasal Embryonic LHRH Factor (NELF)[138], and GPR-54 (kisspeptin receptor) [34, 139] underlie genetic hypogonadotropic hypogonadism due to Gn-RH deficiency. Human cases of Gn-RH deficiency due to the Gn-RH gene mutations have recently been reported [140].

Patients with hypogonadotropic hypogonadism are frequently accompanied by obesity. These include Bardet-Biedl syndrome, Prader Willi syndrome, leptin gene mutation [141, 142], leptin receptor gene mutation [143], and prohormone convertase 1 gene mutation [144, 145]. Frolich syndrome, which is caused by the destruction of ventromedial nucleus of hypothalamus by an invasive tumor, also has both obesity and hypogonadotropic hypogonadism. We describe these diseases in the following section 2.12 Appetite Regulation, Obesity and Anorexia Nervosa. Leptin, not only suppresses the appetite, but also appears to activate the hypothalamo-pituitary gonadal axis. For example, the normal rise of testosterone at onset of puberty in young boys is preceded by a peak of leptin secretion [146]. Moreover, leptin stimulates the secretion of Gn-RH by hypothalamic neurons and gonadotropins by pituitary cells in vitro [147]. Hypogonadotropic hypogonadism is therefore caused by leptin gene mutation [141, 142] or leptin receptor gene mutation [143]. 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 mutations may arise from impaired processing of hypothalamic hormones including Gn-RH and neuropeptides related to the Gn-RH secretion [145].

2.12 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 that 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; it acts on the brain, particularly the hypothalamus, and suppresses appetite and food intake [12, 148]. Furthermore, leptin stimulates the sympathetic nerve activity and controls the metabolic activity centrally. Leptin was originally described as the product of the mouse *obese (ob)* gene [12]. In *ob/ob* mice, obesity was caused by the mutation of the *ob* gene, which results in a lack of circulating leptin. In contrast, the mutation of the leptin receptor gene causes the 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 2.5). 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. 2.15.) and the melanin-concentrating hormone (MCH). (Fig. 2.16).

NPY is a 36 amino acid peptide, originally isolated from porcine brain, and form the pancreatic polypeptide (PP)

Table 2.5 Representative neuropeptides, neurotransmitters and hormones that influence eating behavior.

Stimulate eating	Inhibit eating
Neuropeptide Y	Leptin[b]
Melanin-concentrating hormone	α-MSH
Agouti-related protein	CRH/urocortin
Orexins (hypocretins)	TRH
Ghrelin[a]	Cocaine- and amphetamine-regulated transcript peptide (CART)
Galanin	Peptide YY (3–36)[c]
Opioids	CGRP
Alpha2-noradrenergic	Insulin
GABA	Prolactin-releasing peptide
GH-RH	Somatostatin
Opioid peptides	Cholecystokinin
	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 intestines and acts on the hypothalamus

peptide family together with PP and peptide YY (PYY) [149]. In the peripheral tissues, NPY is localized in the sympathetic nerves, adrenal medulla, etc., 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. 2.15) and paraventricular nucleus [150]. NPY in the infundibular nucleus has been shown to act as a potent stimulator on appetite [151].

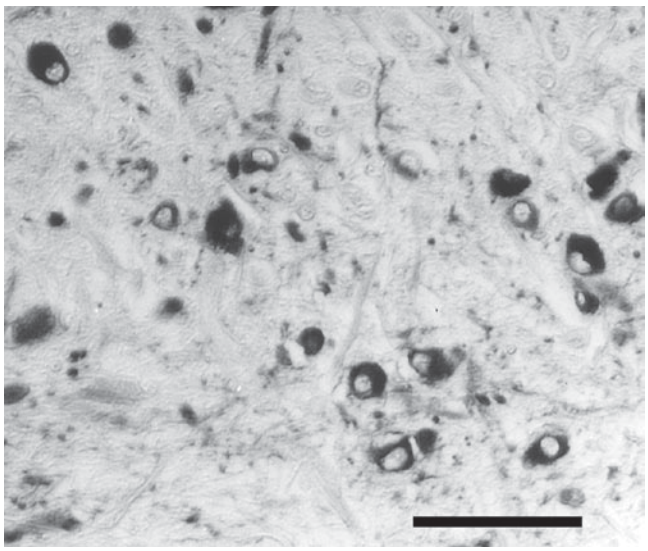

Fig. 2.15 NPY in the human hypothalamus. NPY immunoreactive cell bodies are localized in the infundibular nucleus. Bar = 50 μm

MCH was originally isolated from the salmon pituitary as a skin-color regulating hormone [152]. MCH has an antagonistic action against α-MSH, and make the skin color white by aggregating melanosome within melanophores in fishes. MCH is expressed predominantly in the hypothalamus of the mammals and acts as a neurotransmitter or a neuromodulator [153, 154]. MCH-containing perikarya are found in the posterior and lateral hypothalamic areas, particularly around the mammillary body and fornix (posterior nucleus and perifornical nucleus) (Fig. 2.16). MCH expression was increased in obese mice (*ob/ob* mice) and the central injection of MCH increased the feeding in rats [155]. Recent studies have shown that MCH knockout mice had reduced body weight [156], supporting a role of MCH as an appetite-stimulator. There are at least two subtypes of MCH receptors, MCH receptor 1 and 2, which are distinct from the melanocortin receptors (receptors for ACTH and/or α-MSH) [157–159]. Other appetite-stimulating hormone, orexins (hypocretins) [160, 161] are also expressed in the similar areas of hypothalamus to MCH, but rarely co-localize with MCH in the same neurons [162].

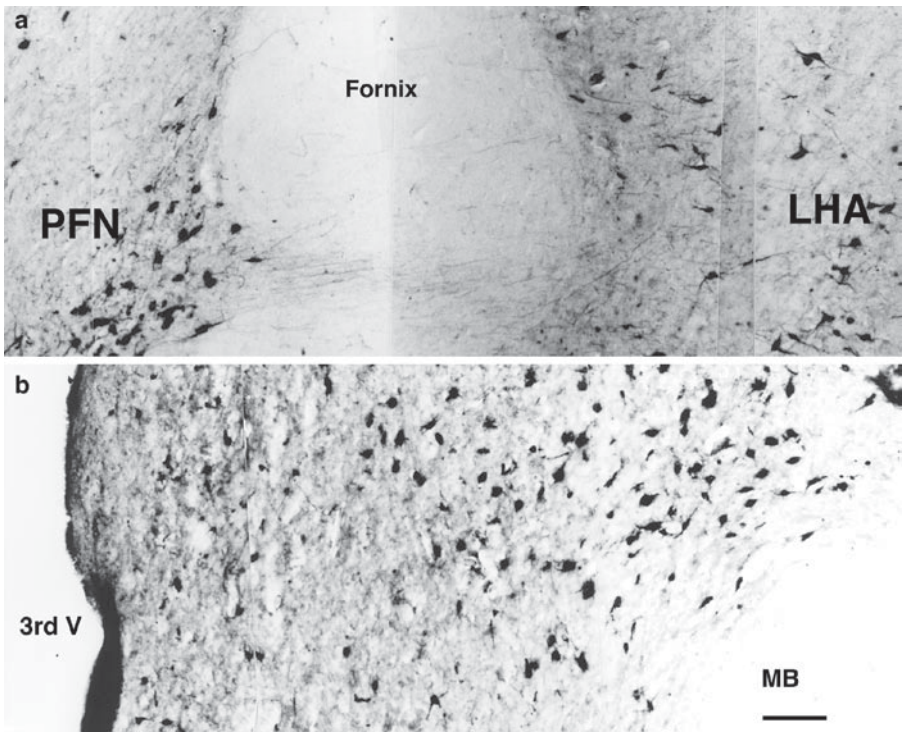

Fig. 2.16 MCH in the human hypothalamus. MCH immunoreactive cell bodies are exclusively localized in the posterior and lateral hypothalamic areas (LHA), including perifornical nucleus (PFN) (**a**) and posterior nucleus (**b**). *3rd V* Third ventricle; *MB* mammillary body. Several photographs are combined. MCH-immunoreactive nerve fibers derived from these hypothalamic neurons are distributed throughout in the brain and pituitary. Bar = 200 μm

α-MSH is generated from the pre-opiomelanocortin (POMC), the common precursor protein with 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 on appetite [163]. 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 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 [164]. 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 [13]. 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 [14, 165]. 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. Blockade of the vagal afferent pathway abolishes ghrelin-induced feeding, indicating that the vagal afferent pathway may be a route conveying orexigenic ghrelin signals to the brain [166]. Moreover, peripheral ghrelin signaling, which travels to the nucleus tractus solitarius (NTS) at least in part via the vagus nerve, increases noradrenaline in the arcuate nucleus of the hypothalamus, thereby stimulating feeding at least partially through alpha-1 and beta-2 noradrenergic receptors [167]. Plasma concentrations of ghrelin are high during fasting whereas they fall to a nadir within an hour of eating [168], suggesting a role of ghrelin in meal initiation.

Figure 2.17 shows the schematic representation of the putative relationship among leptin, ghrelin, and appetite-stimulating and inhibiting neuropeptides in the hypothalamus. 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.

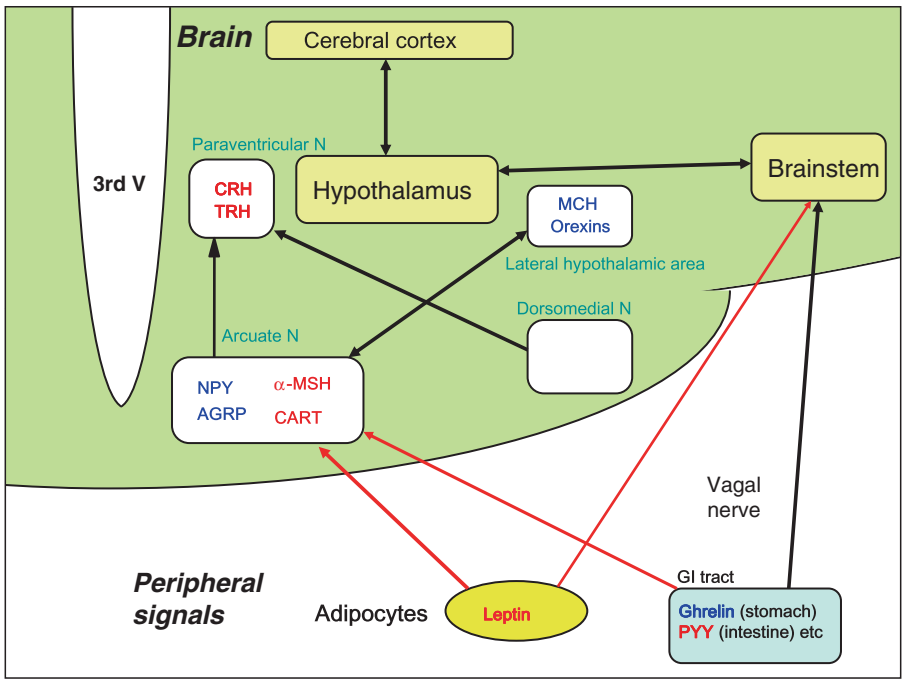

Fig. 2.17 Schematic representation of the putative relationship between peripheral signals (adipokines such as leptin, and gut hormones) and brain (neuropeptides in the hypothalamus, etc.) in the appetite control. Appetite-stimulating and inhibiting factors are shown in *blue* and *red*, respectively. Leptin secreted from adipocytes and gut hormones such as ghrelin and PYY act on brain via the systemic circulation as circulating hormones (shown by *red arrows*) or via the vagal nerve (shown by a *black arrow*). There are complex neural networks among the hypothalamus, the brain stem, the cerebral cortex and other brain areas, and among the nuclei in the hypothalamus. *CART* cocaine- and amphetamine-regulated transcript; *AGRP* Agouti-related protein

Table 2.6 Obesity caused by single gene mutations in human.

Genes	Mechanism to cause obesity	Refs
Melanocortin-4 receptor	Deficiency of α-MSH action to suppress the appetite in the hypothalamus	[173–175]
Melanocortin-3 receptor	Deficiency of α-MSH action to suppress the appetite in the hypothalamus	[176]
POMC	Deficiency of α-MSH secretion in the infundibular nucleus of hypothalamus	[171]
Leptin	Deficiency of functioning leptin secretion by adipocytes	[141, 142]
Leptin receptor	Deficiency of leptin action to suppress the appetite in the hypothalamus	[143]
Prohormone convertase 1	Deficiency of α-MSH processing in the infundibular nucleus of hypothalamus and insulin processing in the pancreatic β-cells (?)	[144, 145]

2.12.1 Genetic Diseases Associated with Obesity Related to Hypothalamus

Obesity is caused under the influence of 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 the appetite regulation in the hypothalamus (Table 2.6). Obesity caused by melanocortin-4 receptor gene mutations is the most prevalent among the monogenic forms of inherited obesity. However, melanocortin-4 receptor gene mutations have been found in only 3–5% of obese patients, indicating that the monogenic forms of inherited obesity are very rare. The vast majority of obesity is considered to be caused by a polygenic disorder. Association of single-nucleotide polymorphisms in the secretogranin III gene with obesity was found in the Japanese populations [169]. Secretogranin III belongs to a family of acidic secretory proteins, known as granins, which are widely expressed in endocrine and neuronal cells, including the hypothalamic neurons and pancreatic β-cells, and participates in the secretion of neuropeptides and peptide hormones.

2.12.1.1 Obesity Due to Single-Gene Mutations

Mutations in the Gene of Leptin or Leptin Receptor

Montague et al. [141] 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. Homozygous frame-shift 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 led to the substitution of an Arg for Trp at position 84 of the mature protein [142]. Although a normal size leptin protein is synthetized in this subject, this is not secreted in serum. Patients with leptin deficiency showed multiple endocrine defects, decreased sympathetic tone, and immune system dysfunction [170]. 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 resulted in a truncated leptin receptor lacking both the transmembrane and the intracellular domains [143]. 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 Gene of the POMC 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 [171]. Patient 1 was found to be a compound heterozygote for two mutations in exon 3 (G7013T, C7133delta) 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 dual role in regulating food intake in the hypothalamus and influencing hair pigmentation, and therefore the deficiency of α-MSH results in obesity and red hair pigmentation. The deficiency in ACTH results in secondary adrenal insufficiency. POMC gene knockout mice lacking the POMC-derived peptides have obesity, defective adrenal development, and altered pigmentation; phenotypes similar to those of the human POMC-deficient patients [172].

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 frame shift mutations in melanocortin-4 receptor gene that caused an early onset in the dominant form of obesity [173]. Subsequent studies have shown that melanocortin-4 receptor gene mutations are a frequent but heterogeneous genetic cause of morbid obesity [174, 175]. 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 [176].

Mutations in the Gene of Prohormone Convertase 1

Prohormone convertase 1 is the endopeptidase which is expressed in the 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, hypocortisolism, and elevated plasma proinsulin and POMC concentrations but very low plasma levels of insulin and ACTH [144]. The 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 [145]

2.12.1.2 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 [177]. This disorder is heterogenous and at least four gene loci responsible for this disorder (BBS1-4) 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 [178]. 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 [179]. These include short statue, muscular hypotonia, excessive appetite with progressive obesity, hypogonadism, mental retardation, behavioral abnormalities, sleep disturbances (including sleep apnea), and dysmorphic features. Prader-Willi syndrome is the most frequent cause of syndromic obesity occurring in one in 10,000–25,000 births. 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.

The 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 [180]. There were a 30% reduction in GH-RH-releasing neurons in the arcuate nucleus, a down-regulation of NPY, and a deficiency in vasopressin [181]. Magnetic resonance imaging 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 [182]. Plasma ghrelin levels in children with Prader-Willi syndrome are elevated [183], and may be related to the pathogenesis of abnormal appetite control.

2.12.2 Obesity Due to Non-genetic Hypothalamic Causes

2.12.2.1 Frolich's Syndrome

Frolich's syndrome (adiposogenial dystrophy) was originally characterized as delayed puberty, hypogonadism, and obesity associated with a tumor that impinges on the hypothalamus [184]. Several organic lesions of the hypothalamus, however, can cause this disorder, including tumors, encephalitis, microcephaly, Friedreich's ataxia, and demyelinating diseases.

2.12.3 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, a body image disturbance, and amenorrhea [185]. The etiology of this disorder is unknown. It occurs most often in young women.

Multiple endocrine disturbances and hypothalamic dysfunction are known to occur in patients with anorexia nervosa, which are as follows. 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 the feeding and the LH secretion.

Plasma GH levels are elevated whereas plasma levels of insulin-like growth factor I are decreased. Fasting plasma ghrelin levels were significantly higher in patients with anorexia nervosa than in normal age-matched female controls

[186]. Therapeutic intervention in a psychosomatic institution caused a BMI increase and a significant decrease in circulating ghrelin levels. Thus, elevated plasma ghrelin may be a cause for the elevated plasma GH levels, and ghrelin resistance on the appetite may be present in cachectic states.

Plasma levels of leptin are reduced with low weight and the percentage of body fat in subjects with anorexia nervosa [187]. It has been shown that leptin has a stimulatory action on hypothalamo-pituitary-gonadal axis [188], raising the possibility that hypogonadism associated with anorexia nervosa is partly due to the leptin deficiency. On the other hand, the hypothalamic dysfunction with multiple endocrine disturbances seen in anorexia nervosa may be secondary phenomena due to the unknown central disturbance.

2.13 Narcolepsy and Orexins (Hypocretins)

Narcolepsy is a disabling neurological disorder that affects more than 1 in 2,000 individuals. This disorder is characterized by daytime sleepiness, sleep fragmentation, and symptoms of abnormal REM sleep, such as cataplexy, sleep paralysis, and hypnagogic hallucinations [189]. Most human cases of narcolepsy occur sporadically and the disorder is generally believed to be multigenic and environmentally influenced, whereas in canine (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 tight HLA association, the human narcolepsy was suggested to be autoimmune in nature. Recent studies have shown that neuropeptides, orexins (hypocretins), are involved in the pathogenesis of narcolepsy [189–192].

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 [160, 161]. The orexins are named following their central appetite-stimulating action. These peptides are specifically expressed in the hypothalamus, and the positive cell bodies are mostly 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 [189]. 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 [190]. In most human cases of narcolepsy, orexin levels in the cerebrospinal fluid have been shown to be decreased to undetectable levels [191]. 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 [192]. On the other hand, one orexin mutation, impairing peptide trafficking and processing, was found in a single case with early onset of 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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Chapter 3
Tumors in the Adenohypophysis

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3.1 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 approximately 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 have undergone considerable change in the last three decades of the twentieth 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 [67]. 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 vassopressin and oxytocin and consists of terminations of nerve fibers arising in the supraoptic land 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

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via the unmyelinated nerve fibers to the posterior lobe, where they are stored in the neurosecretory granules until subsequently released [2].

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 land. In 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.

3.2 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 approximately 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. 3.1).

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

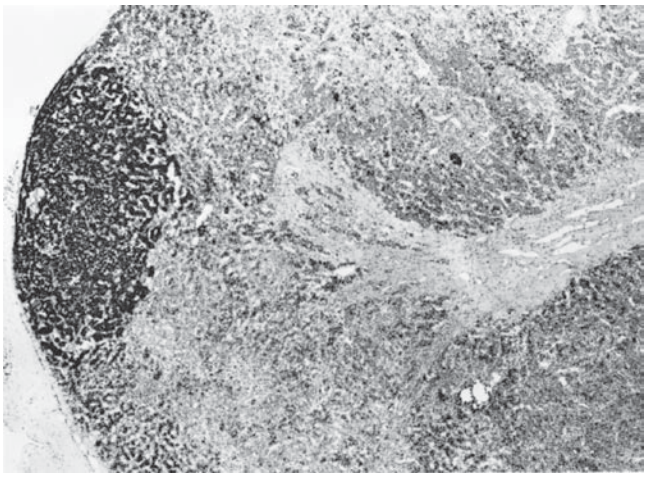

Fig. 3.1 Incidental microadenomas are a frequent finding in the elderly. They are benign, well-demarcated tumors, which either lack immunoreactivity for pituitary hormones or stain mainly for prolactin as is shown in the picture. Original magnification ×40

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 [3].

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 [4–7]. 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 [8, 9]. This approach takes into consideration the clinical and laboratory findings, neuroimaging findings as well as histologic, immunocytochemical, imaging, and ultrastructural features.

3.3 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, 10]. 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 land hormone levels in both basal and dynamic states. Such measurements are sensitive diagnostic indicators in the approximately 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 hypesecretion 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 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 interior and superior hypophysial arteries, both branches of the internal carotid arteries [11]. 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 infandibulum. 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. Since 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 [12, 13].

3.4 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 microadenomas. In this regard, the radiologic classification of Hardy is easily applied and clinically useful [14]. 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 11, 111, and IV, respectively. Macroadenomas are further subclassified on the basis of their extrasellar extensions, whether suprasellar, parasellar, inferior, or a combination of these.

3.5 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 land can readily be made. This is of obvious importance, but can be challenging, given the small and fragmented nature of many specimens obtained transphenoidally. After exclusion of normal compressed or hyperplastic adenohypophysial tissue the nature of the adenoma has to be assessed [15–18].

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 anastamosing 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 [16, 18].

Pituitary adenomas are usually well demarcated and consist of compressed nontumorous adenohypophysis (by a pseudo-capsule) 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 expansion (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 the 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 findings of 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 as 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 correlate 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 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 stain is also preferred to the demonstration of pituitary hyperplasia, as its main morphological feature is the expansion of acini (Fig. 3.2).

3.6 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 [19, 20]

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 maybe 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

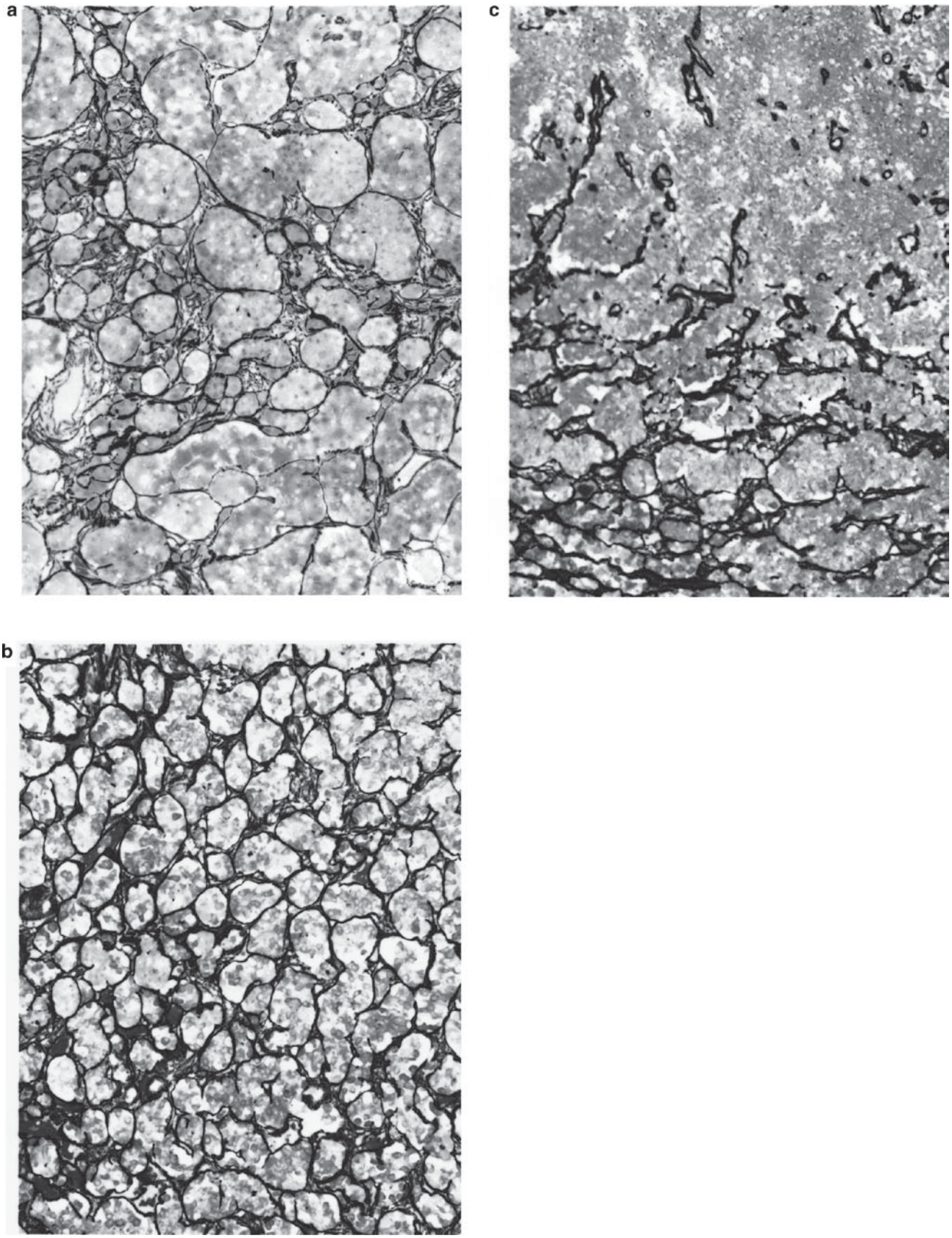

Fig. 3.2 The Gordon-Sweet silver technique for reticulin staining is useful in distinguishing hyperplasia (**a**) from normal adenohypophysial tissue (**b**) or from adenomas (**c**). While normal adenohypophysis (**b**) displays a regular network of delicate reticulin fibers, hyperplasia (**a**) causes enlargement, distortion, and confluence of acini. In contrast, loss of acinar structure is evident in pituitary tumors (**c**). Original magnification ×100

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.

3.7 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 [21]. As a result, it was pivotal in the establishment of a functional classification of pituitary adenomas and in their ultra-structural 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 approximately 50% of adenohypophysial cells and are located mainly in the "lateral wings" of the PD. Somatotroph adenomas generally arise at this site. Lactotroph represent 10–25% of adenohypophysial cells and are maximally concentrated in the posterior aspect of the lateral wing just anterior to the neural lobe. Most lactotroph adenomas originate in this area. Corticotrophs represent adenomas hypophysial 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. 3.3).

3.8 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 [16, 18, 20, 22, 23]. A shortcoming of ultrastructural investigation relates to small sample size, which introduces the possibility of "sampling error."

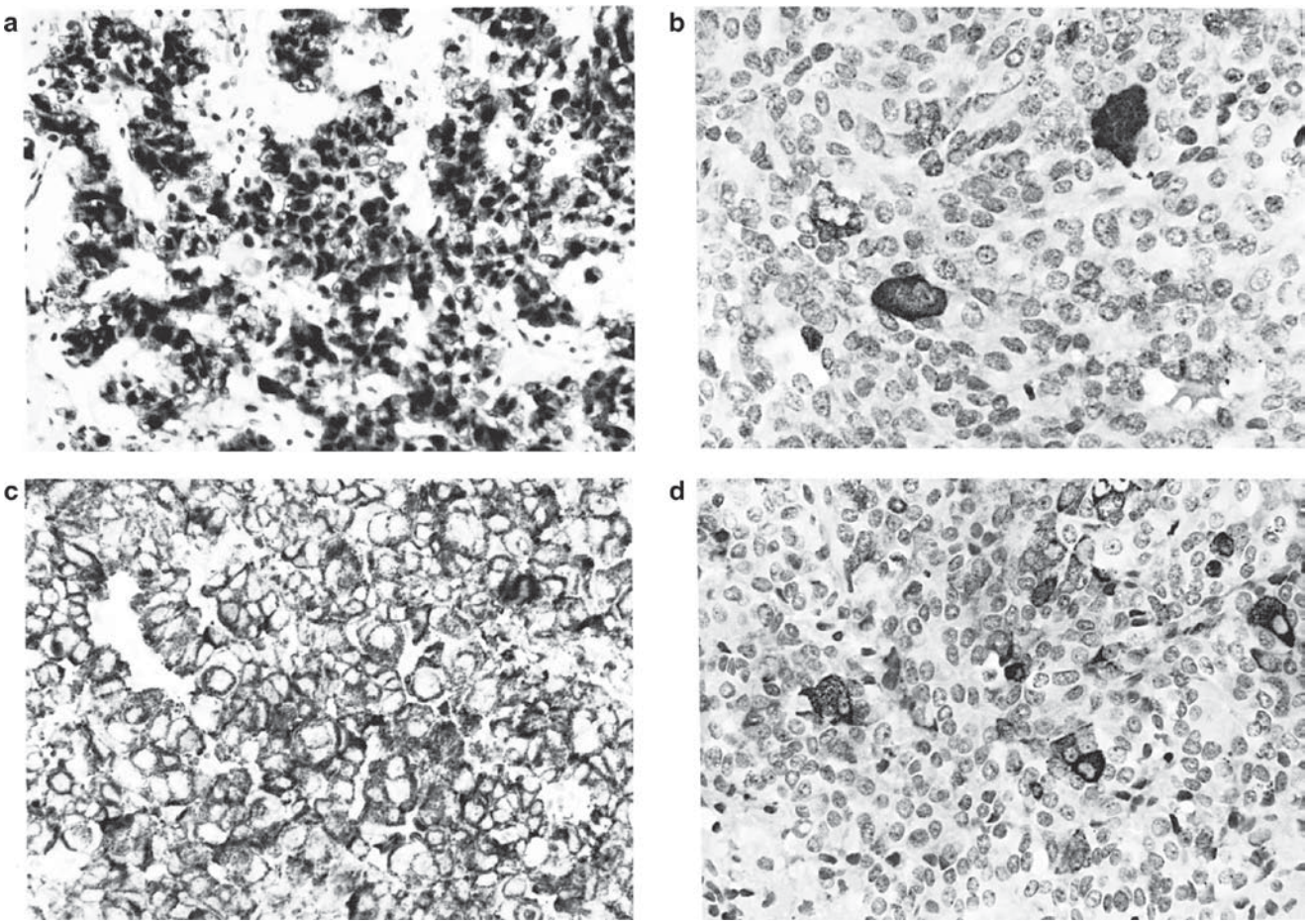

Fig. 3.3 In some adenoma types immunostaining for specific adenohypophysial hormones may not be sufficient for conclusive diagnosis; it may even be misleading. In such cases electron microscopy is mandatory for correct diagnosis. (**a**) Immunoreactivity for PRL in a prolactin cell adenoma showing the 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. 3.4) showing intense ACTH immunostaining in the cytoplasm of adenoma cells. (**d**) In gonadtroph adenomas of the female type (see also Fig. 3.5) a light, scattered immunostaining for ACTH may be apparent. Original magnification ×400

3.9 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, 16, 18, 24]. 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 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.

3.10 Densely Granulated GH Cell Adenoma

These tumors comprise what has been termed the “classic acidophilic adenoma of acromegaly.” It accounts for approximately 8% of all pituitary adenomas and is characterized by a relatively slow growth rate, limited invasiveness, and an overall indolent biologic. 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 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.

3.11 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, more rapidly growing and less responsive to somatostatin analog treatment* [25]. 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 ail admixture of intermediate (cytokeratin) filaments and smooth endoplasmic reticulum (SER), is located in the Golgi region, and often indents the nucleus [26].

In GH cell adenomas treated with long-acting somatostatin analogs, mild cell shrinkage, accumulation of lysosomes, and interstitial as well as perivascular fibrosis can often be seen. These alterations are inconsistently present and are usually not marked.

3.12 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 [16, 18, 27].

PRL cell I 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. Diffuse cytoplasmic immunostaining for PRL is a feature only in a small minority of cases. Thus, two ultrastructural types of PRL cell adenoma are recognized.

3.13 Sparsely Granulated PRL Cell Adenoma

This is the most frequent tumor type (Fig. 3.4). Its cells have the same striking appearance of hormonal activity as nontumorous PRL cells, abundant RER often in large concentric

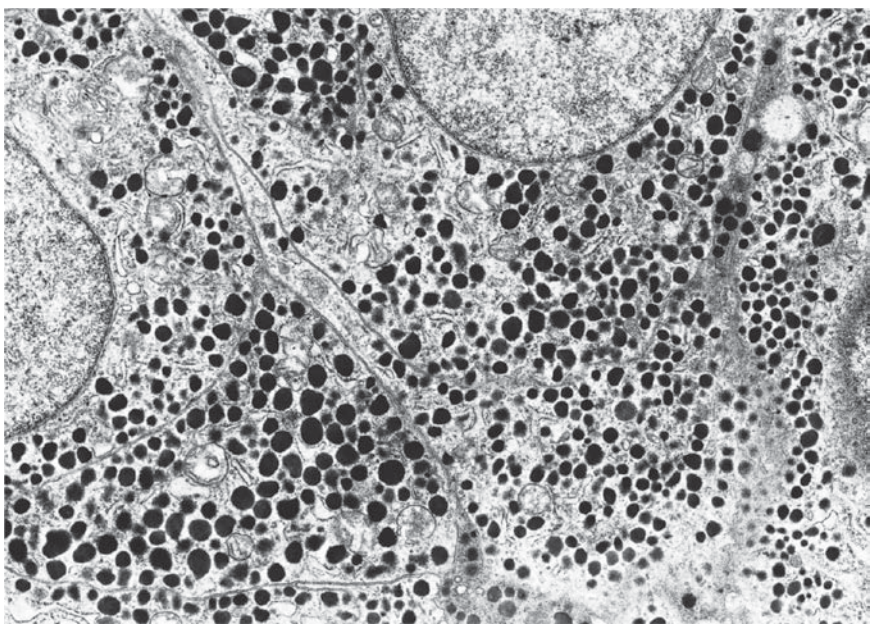

Fig. 3.4 Corticotroph adenoma. The cytoplasm contains numerous secretory granules displaying characteristic morphology. Original magnification ×11,760

whorls, and prominence of the Golgi apparatus. The latter often contains pleomorphic, immature secretory granules. Secterory 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.

3.14 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 PR cell adenomas contain abundant cytoplasmic secretory granules, thus their acidophilic appearance on H&E stain and diffuse use cytoplasmic PRL immunopositivty. Secterory granule 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 rumors 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.

3.15 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-related peptides in the cytoplasm of adenoma cells is [16, 18, 28]. 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. 3.5). The other characteristic ultrastructural marker is bundles of keratin immunoreactive intermediate filaments disposed around the nucleus [2, 29, 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 the high levels of cortisol are not a feature of Nelson's syndrome, and because Crooke's hyaline change is the negative feedback

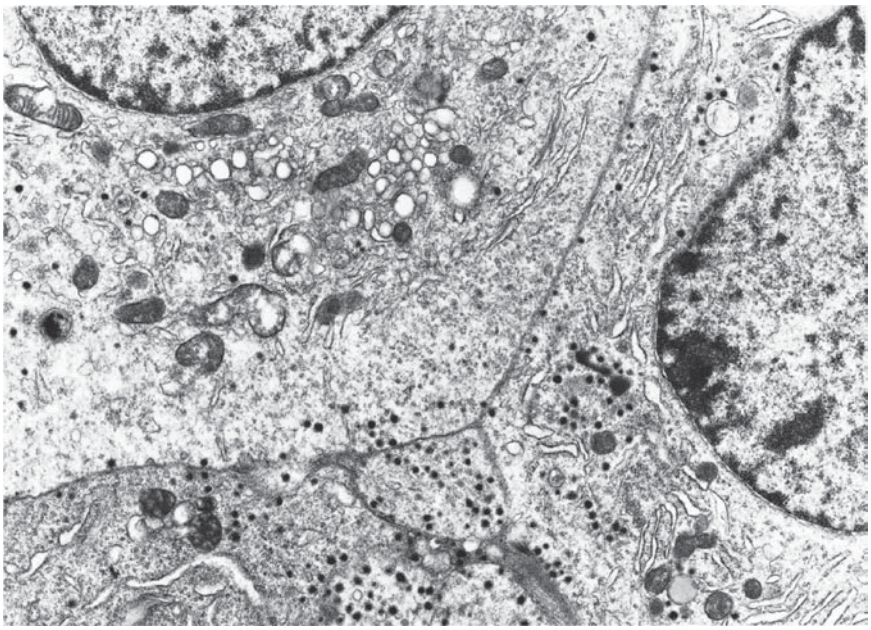

Fig. 3.5 Gonadotroph adenomas of the female type comprise polar cells with long processes containing most of the small secretory granules. The Golgi complex shows vacuolar transformation (honeycomb Golgi). Original magnification ×13,640

effect of elevated glucocorticoid levels, intermediate filaments lack in Nelson's adenomas.

3.16 TSH Cell Adenoma

This tumor is rare, representing only about I % 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 in appropriately 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.

3.17 FSH/LH Cell Adenoma

Gonadotroph adenomas may be associated with increased serum levels of FSH, LH, and/or u-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 [16, 18, 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, vary in 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.

3.18 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 that are 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 [16, 18, 36, 37].

3.18.1 Silent "Corticotroph" Adenoma Subtype I

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 I 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.

3.18.2 Silent "Corticotroph" Adenoma Subtype 2

This primarily affects men. Histologically, most are chromophobic and, in contrast to subtype I "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 sub-type I adenomas, and lack intermediate filaments. On the other hand, the secretory granules are similar to those of Cushing's and silent subtype 1 adenomas.

3.18.3 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 immunoreactive. 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.

3.19 Null Cell Adenomas

Null cell adenomas are mainly *found* 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, 16, 18]. 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 secretory 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.

3.20 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 [38–40]. 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 Chaps. 4 and 5.

3.21 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 [41, 42].

3.22 Hypothalamic Factors and Pituitary Tumors

Despite having demonstrated the clonality of most if not all pituitary tumors, a contribution of hypothalamic hormones to pituitary tumorigenesis has been considered [43]. For good reason there is renewed interest in integrating their role in the current multi-step monoclonal model. For example, it has been demonstrated that the 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 [44, 45]. Animal models also provide support for the notion. For example, rats transgenic for GH-RH develop somatotroph hyperplasia and subsequently pituitary adenoma. It has also been shown that the dopamine receptor (D2) knockout rodents develop PRL-producing pituitary adenomas [46, 47, 68].

3.23 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, 48]. 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 the protracted estrogen stimulation contributes to transformation and/or neoplastic progression of PRL cell adenomas in the rodent and human pituitaries.

3.24 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 [49–51]. 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, mutaion 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, nm23, 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.

3.25 Plurihornomanility

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. 3.6) [52, 53]. 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 [54, 55].

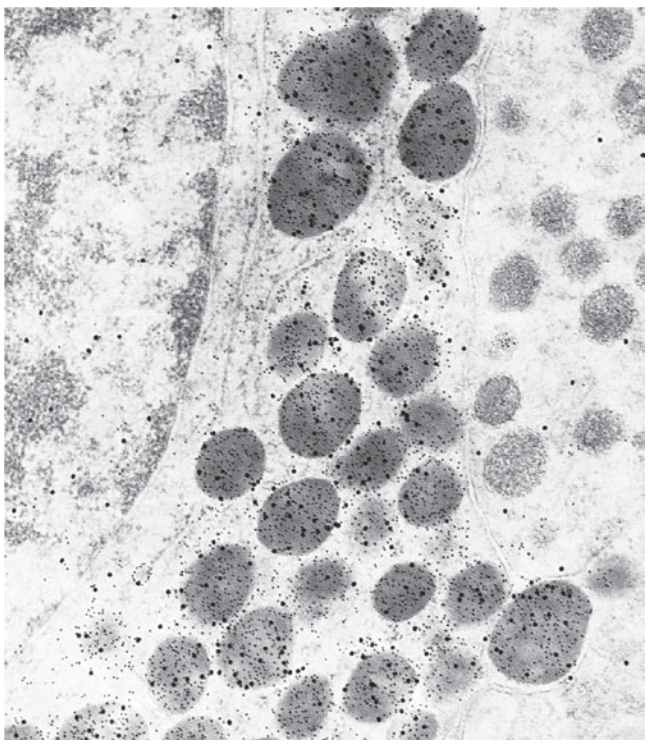

Fig. 3.6 Double-immunogold method demonstrates colocalization of GH (immunolabeling with 10-nm gold particles) and PRL (immunolabeling with 20-nm gold particles) in the same secretory granules of mammosomatotroph in a bihormonal tumor. Original magnification ×31,160

It is not clear whether plurihormonal cells occur more frequently in adenomatous or in normal, nontumorous pituitaries [56]. 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 [57–59].

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 with acromegaly produce GH and one or more glycoprotein hormones, primarily α-subunit [60, 61]. These patients have acromegaly, elevated serum GH and insulin-like growth factor (IGF)-I 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, 16, 18].

3.26 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.

3.27 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 from of giant mitochondria. The sparse, randomly distributed secretory granules are small (50–200 nm) [62].

3.28 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.

3.29 Cell Proliferation Markers

Several cell proliferation markers including proliferative cell nuclear antigen (PCNA) MIB-1 I (Ki-67), p-27, cyclins, topoisomerase II-α, AGNOR (argyropilic nuclear organization region), and BrdUrd (bromodeoxyuridine) can be used to document kinetic abnormalities that play a role in tumor progression (63–65). As detected by the MIB-1 I 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 the 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 show in 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. 3.7). 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 the microvessel density. An emerging marker is MGMT (O6-methylguanine-methyltransferase) whose lack of staining in pituitary tumors predicts responsiveness to treatment with temozolomide [66]

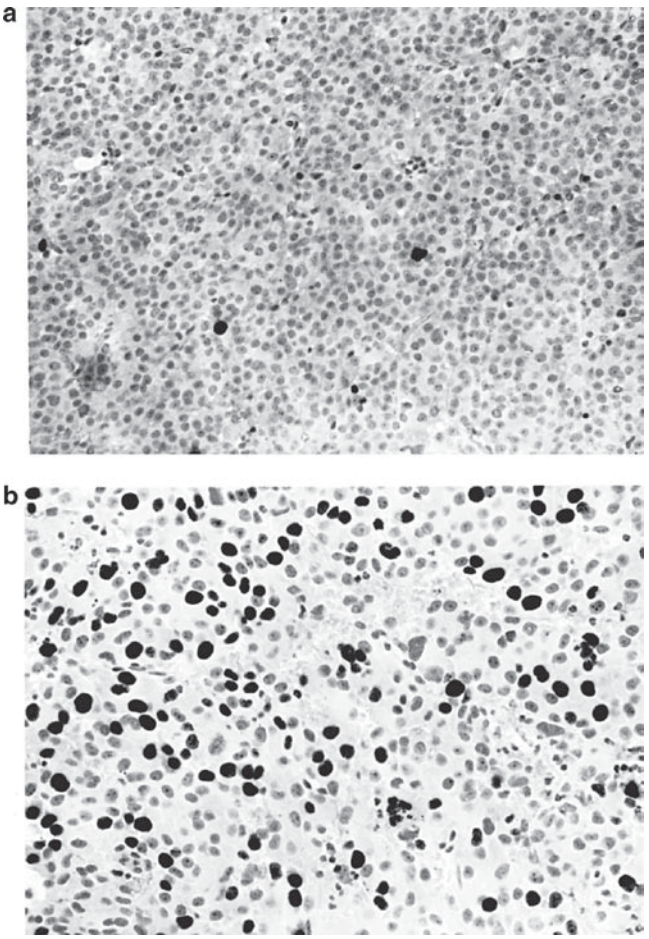

Fig. 3.7 Although pituitary tumors generally exhibit a slow growth rate previous studies have shown a relationsionship between the expression of cell-proliferation markers and aggressive tumor behavior. (**a**) Pituitay tumors showing a low (<1%) MIB-1 labeling index. (**b**) Pituitary tumor showing high (>7%) MIB-1 labeling index. Original magnification ×250

3.30 Atypical Pituitary Neoplasms and Pituitary Carcinomas

The diagnosis of pituitary carcinoma presents a challenge. Based on the current WHO classification of pituitary neoplasms, documentation of metastasis is required for diagnosis of pituitary carcinoma. However, tumors demonstrating invasive growth, increased mitotic index, Ki-67 labeling index ≥ 3% and extensive nuclear reactivity for p53 are termed "atypical pituitary neoplasms" [8, 9]. Pituitary carcinomas are very rare and are defined as primary neoplasms of the adenohypophysis that undergo craniospinal and/or systemic spread [4–7]. 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

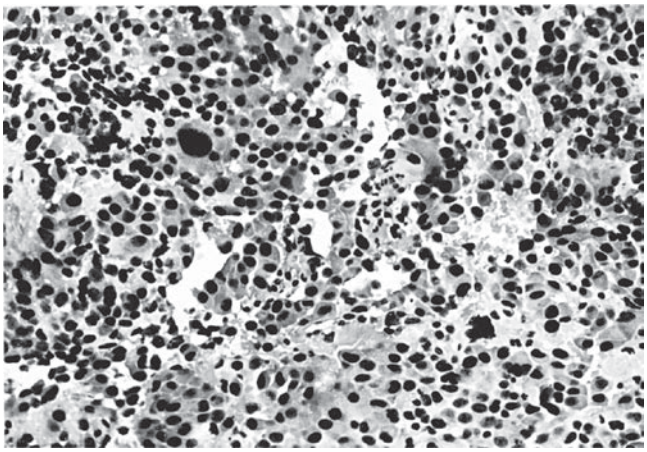

Fig. 3.8 Pituitary carcinoma showing nuclear and cellular atypical. Original magnification ×250

increased numbers of mitotic figures, nuclear atypia, hyperchromasia, pleomorphism, nucleolar prominence, and necrosis (Fig. 3.8). 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.

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

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4.1 Introduction

The pituitary gland is derived from the oral epithelium as primordium which later gives rise to an infolding called Rathke's pouch. The Rathke's pouch is composed of ventral and dorsal limbs. The former develops to form various hormone-producing cells, growth hormone (GH), prolactin (PRL), thyrotropin-stimulating hormone (TSH), adrenocorticotropic hormone (ACTH), follicle-stimulating hormone (FSH), and luteinizing hormone (LH). On the other hand, the dorsal limb produces only ACTH and melanocyte-stimulating hormone (MSH), both processed from proopiomelanocortin (POMC) (Fig. 4.1a–g).

The embryogenesis and functional differentiation of the pituitary glands have been studied for more than half a century. It has been presented as an essential model to study functional differentiation in organogenesis by the pituitary gland. Recent development in molecular technologies and the cloning of a series of transcription factors which was regulated by several signal transductions have stimulated rapid progress in the clarification regarding the involvement of the pituitary cells in hormone production.

This chapter emphasizes the development of the pituitary glands and the roles of transcription factors and cofactors, including several signal transduction molecules.

4.2 Early Development of the Pituitary Glands

It has been shown that the ventral limb of the Rathke's pouch differentiates in a particular chronological order and forms the anterior lobe (Fig. 4.2a). It is well known that between αSU (glycoprotein alpha-subunit) and POMC are found the first diverging points in the development of the ventral limb, and are followed by GH, PRL, TSH, FSH, and LH. Among these, TSH, FSH, and LH are glycoproteins which are composed of common αSU and morpho-functionally specific βSU. On the other hand, the dorsal limb of the Rathke's pouch develops into a "band-like" structure, with predominant differentiation into αSU and forms an intermediate lobe. POMC (ACTH) in the anterior lobe appears earlier than that in the intermediate lobe.

In the human pituitary gland, similarly, the ventral limb develops into various hormone-producing cells (Fig. 4.2b). And it is of particular interest to note that the dorsal limb forms an intermediate lobe-like structure only during fetal life. In the adult pituitary, these dorsal limb-derived cells remain as "invading anterior cells" in the posterior lobe (Fig. 4.2c) [1]. As in the intermediate lobe in rodents, these "invading anterior cells" differentiate predominantly to POMC (ACTH); however, immunohistochemical αSU positivity is rather rare (Fig. 4.1h).

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 hormone-producing cells show specific intimate relationship, i.e. ACTH-GH, FSH/LH-PRL (especially in females). The POMC cells show cytoplasmic processes directed to the capillaries and also show cytoplasmic attachment to the GH cells. Another unique finding in the human pituitary gland is that GH-producing cells are also immunohistochemically positive for αSU and PRL.

Folliculo-stellate (FS) cells are unique cells in the anterior pituitary, and are marked by the presence of S100 protein and are easily detected by immunohistochemistry (Fig. 4.1i). The FS cells are particularly specific in their shape because of a few elongated cytoplasmic processes which engulf the other hormone-producing cells and produce the cytokine [2–4].

4.3 Development of Hypothalamo-Pituitary Axis

It has been well known that the functions of the anterior pituitary cells are regulated by hypothalamic hormones, as indicated, i.e. GH-releasing hormone (GHRH) → GH

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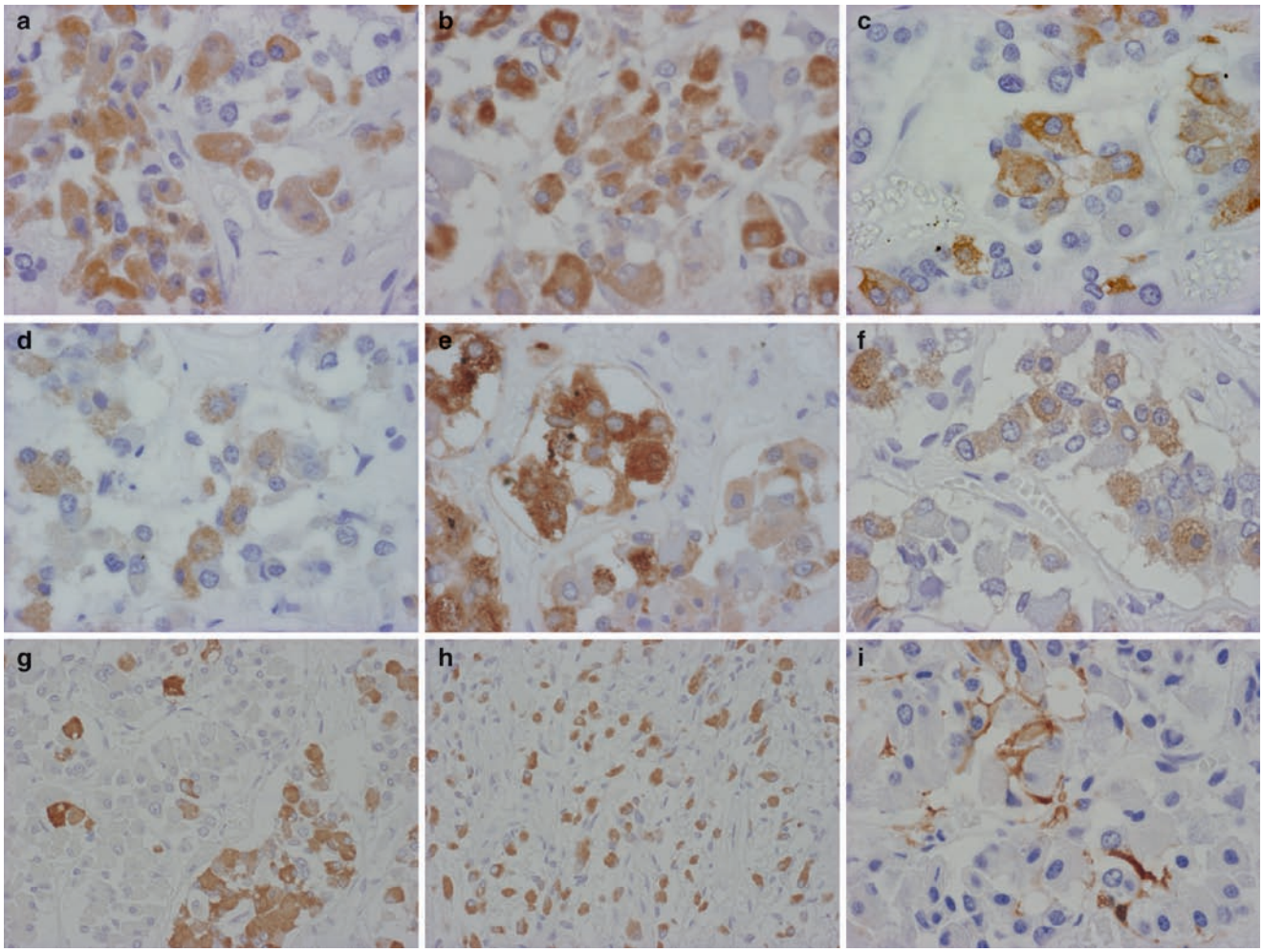


Fig. 4.1 Immunohistochemical localization of pituitary hormones and S100 protein as a marker of foliculo-stellate (FS) cells in human pituitary. (**a**) GH-producing cells; somatotropes, (**b**) PRL-producing cells; lactotropes, (**c**) TSH-producing cells; thyrotropes, (**d**) LH-producing cells and (**e**) FSH-producing cells; gonadotropes, (**f**) αSU (TSH , FSH, and LH are composed of common αSU and functionally specific βSU), (**g**) ACTH-producing cells; corticotropes, (**h**) invading anterior region, (**i**) S100 positive foliculo-stellate (FS) cells. Brown; hormones and S100, Blue; hematoxylin nuclear staining

cells, dopamine, prolactin-releasing peptide (PrRP) → PRL cells, thyrotropin-releasing hormone (TRH) → TSH cells, corticotropin-releasing hormone (CRH) → POMC cells, gonadotropin-releasing hormone (GnRH) → FSH/LH cells (Fig. 4.3). These anterior pituitary cells possess receptors (Rs) for these factors from the hypothalamus, i.e. GH cells – GHRH-R, PRL cells – dopamine receptor (D2R), PrRP-R, TSH cells – TRH-R, POMC cells – CRH-R, FSH/LH cells – GnRH-R. PRL cells are also known to possess estrogen receptor (ER), which binds to DNA. In rodents, the developmental relationship between hypothalamic factors and pituitary hormones has been studied by immunohistochemistry (IHC) and in situ hybridization (ISH). It is of functional and morphological interest that the hypothalamic factors and these receptors such as GHRH/GHRH-R, and CRH/CRH-R appear before corresponding hormones GH and POMC, respectively. The reason for this reciprocal appearance remains to be further investigated.

4.4 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 corresponding receptors on the cell membrane and in the nuclei. The transcription factors are divided into two groups: (1) transcription factors in early pituitary development and (2) transcription factors in the

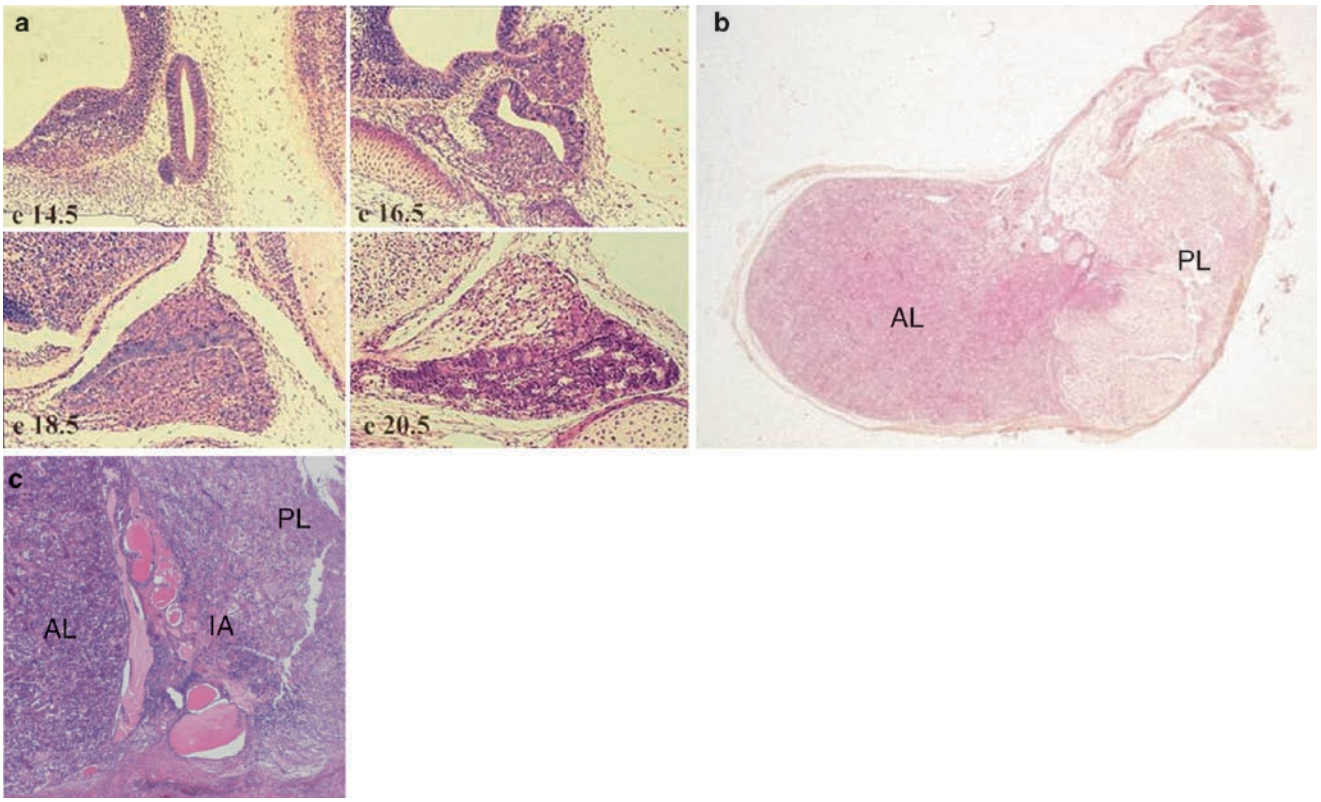


Fig. 4.2 Human and fetal rat pituitary glands. (**a**) Development of the rat pituitary gland: Structural development of pituitary stars from the Rathke's pouch which was derived from the oral ectoderm. It is known that the anterior limb of the Rathke's pouch develops into the anterior lobe and the posterior limb into the intermediate lobe. (Embryonic days 14.5–20.5). (**b**) Human pituitary gland: In human pituitary, during fetal development, definite intermediate structure exists and produces ACTH and αMSH. (**c**) But it changes its structures to the "invading anterior cells" after birth. In human pituitary, ACTH positive cells which are located peripherally in early development migrate to the center of the glands. *AL* anterior lobe, *PL* posterior lobe, and *IA* invading anterior pituitary region

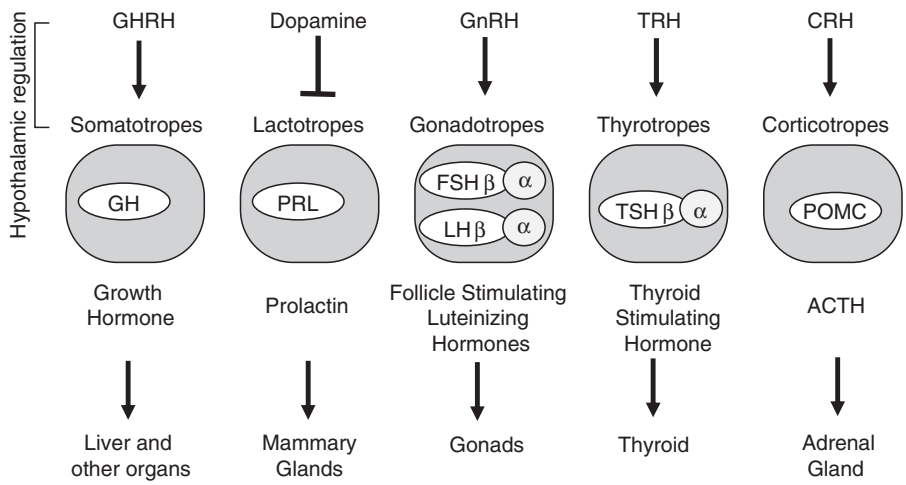


Fig. 4.3 Pituitary hormone productions are regulated by hypothalamic hormones and feedback

later functional differentiation (Table 4.1). The study of transcription factors was first stimulated by cloning pituitary-specific transcription factor-1 (Pit1/GHF1) and has resulted in the discovery of many subsequently cloned transcription factors, both in the early development and in the later functional stages. (3) Cofactors, which work synergistically with transcription factors, also play critical roles as transcription regulator in pituitary hormone expression.

Table 4.1 Transcription factors in early or later stages of pituitary development

Involved in early pituitary development
Hesx1/Rpx Lhx3 Lhx4 Ptx1 Ptx2 Prop1
Involved in later pituitary differentiation
Pit1 GATA2 NeuroD1 Tpit SF1 ER

4.4.1 Transcription Factors in Early Pituitary Development (Table 4.1, 4.2)

Rpx/Hesx1 is the factor which regulates the formation of Rathke's pouch and Lhx3 (pLim, Lim3) is the factor which 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 for differentiation towards POMC, but is currently considered to be another transcription factor which appears in the early development and collaborates with the following more functionally oriented transcription factors, i.e. Pit1, NeuroD1, and Tpit [5–8]. Ptx1 and Ptx2/Pitx2 express in primordial Rathke's pouch in oral epithelium and throughout the development of pituitary. In the null mutant mice of Ptx1, it has been reported that the development of the anterior pituitary gland is deficient and the pituitary cells reduce the expression of TSHβ, LHβ, FSHβ, and αSU but the expressions of GH and POMC are unchanged [9]. Further, Ptx2 expression overlaps with Ptx1 in early developing pituitary. In the pituitary of the null mutant of Ptx2, invagination occurs normally but subsequent development is reduced. In the pituitary, Hesx1 is not expressed but Lhx3 is expressed. The phenotype is similar to that of $Lhx3^{+/-}$ and $Lhx4^{-/-}$, and the relative roles of Ptx1 and Ptx2 may be similar to those of Lhx3 and Lhx4 (Fig. 4.4a) [7, 10]. Forkhead transcription factor, Foxl2 co-localizes with glycoprotein hormone αSU in quiescent cells of the mouse pituitary from embryonic d 11.5 through adulthood. Foxl2 controls the hierarchy of pituitary development and regulates αSU gene expression [11].

4.4.2 Transcription Factors in the Later Functional Differentiation (Tables 4.1 and 4.2)

Pit1 is a transcription factor which regulates the functional differentiation of the anterior pituitary cells to GH, PRL, and TSH. Pit1 has six exons which code proteins of 291 amid acid sequence and a molecular weight of 33kDa. Pit1 has two isoforms with insertions of 26 amino acids (Pit1β) and 14 amino acids (Pit1T), respectively (Fig. 4.5) [12]. The upstream promoter region of GH gene has two Pit1 binding sites. The PRL and TSH genes contain eight five Pit1 binding respectively (Fig. 4.6). NeuroD1 is well documented to show to function with Ptx1 whose binding site is located near that of NeuroD1 [13]. GATA2 is one of the GATA-binding transcription factor family that plays a role with Pit1 in the differentiation of TSH [14]. Steroidogenic factor-1 (SF1) is a key factor for the differentiation of LH. DAX1 has been regarded as a factor for LH, but recent study has revealed its presence in all kinds of pituitary cells.

Table 4.2 Major transcription factors involved in the differentiation of anterior pituitary gland

	Other names	DNA binding domain	Related roles
Egr1	KROX24, NGFIA	Zinc finger	Development of gonadotropes
ERα	ESR1	Nuclear receptor	Regulation of PRL, LH, FSH expression, and proliferation of lactotropes
FOXL2	P-Frk	Forkhead box	Involved in αSU gene expression
GATA2	NF-E1b	Zinc finger	Differentiation of gonadotropes and thyrotropes
Lhx3	pLIM, Lim3	LIM-HD	Development of anterior and intermediate pituitary, and differentiation of GH-, PRL-, TSH-, LH-, FSH-producing cells
Lhx4	GSH4	LIM-HD	Development of anterior and intermediate pituitary
NeuroD1	BETA2	bHLH	Regulation of POMC gene expression in corticotropes
Pit1	GHF1, POU1F1	POU-HD	Differentiation of GH-, PRL-, TSH-producing cells
Prop1		Paired-like HD	Important in GH-, PRL-, TSH-, LH-, FSH-producing cells differentiation
Ptx1	Pitx1, P-OTX	Bicoid-related HD	Important pituitary occurrence, and activation of several hormone genes in later development
Ptx2	Pitx2, RIEG	Bicoid-related HD	Important pituitary occurrence, and activation of several hormone genes in later development
Hesx1	Rpx	Paired-like HD	Important in early pituitary development
SF1	Ad4BP, Nr5a1	Nuclear receptor	Required in LH-, FSH-producing cells
Tpit	Tbx19	T box	Regulation of POMC gene expression in corticotropes and meleanotropes

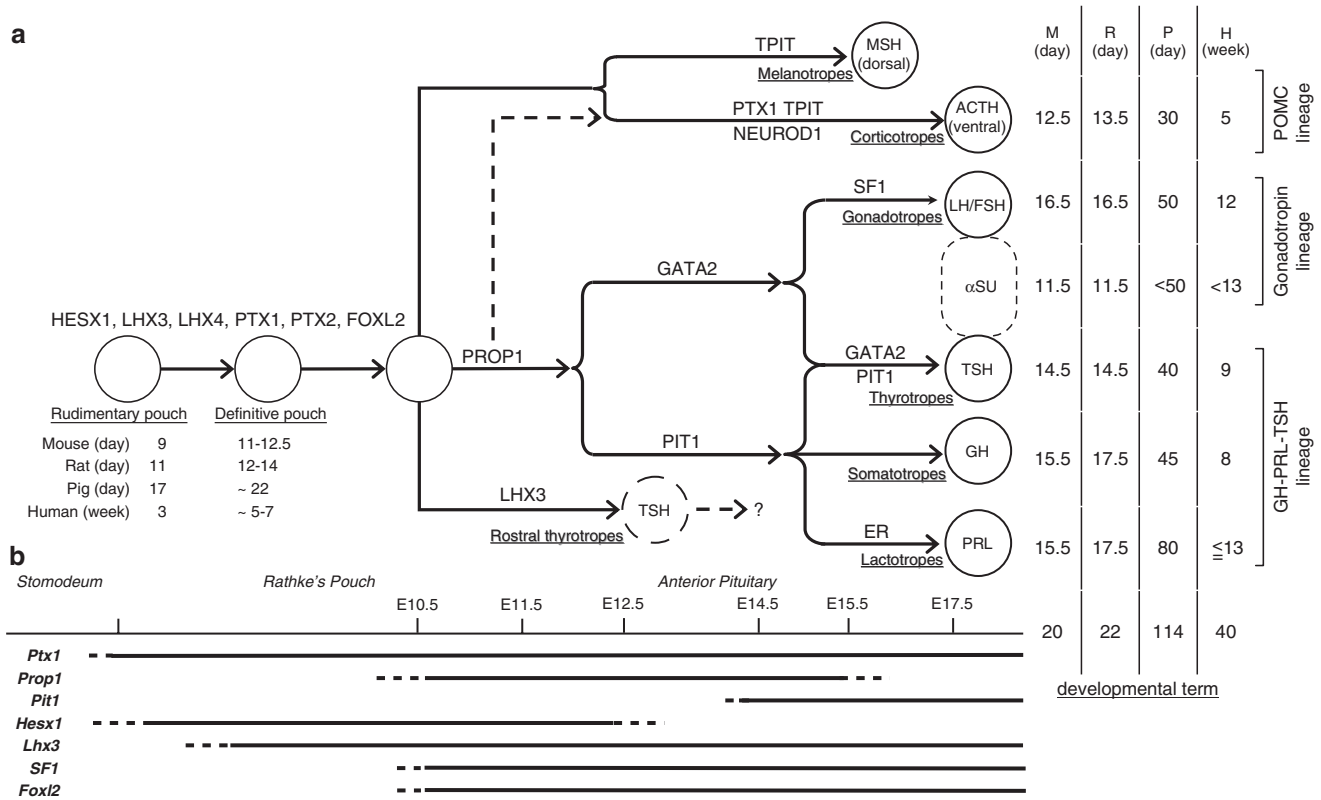


Fig. 4.4 Transcriptional regulation of pituitary development in mammals. (**a**) Several transcription factors play critical roles for functional differentiation into specific hormone-producing cell lineages, including POMC lineage (corticotropes), gonadotropin lineage (gonadotropes), and GH-PRL-TSH lineage (somatotropes, lactotropes, thyrotropes). The approximate developmental terms (in days or weeks of gestation) can be observed in four mammals. (**b**) The approximate timing of mRNA expression in the mouse pituitary is shown for transcription factors. (modified after Scully et al. 2002 and Savage et al. 2003 [18, 49])

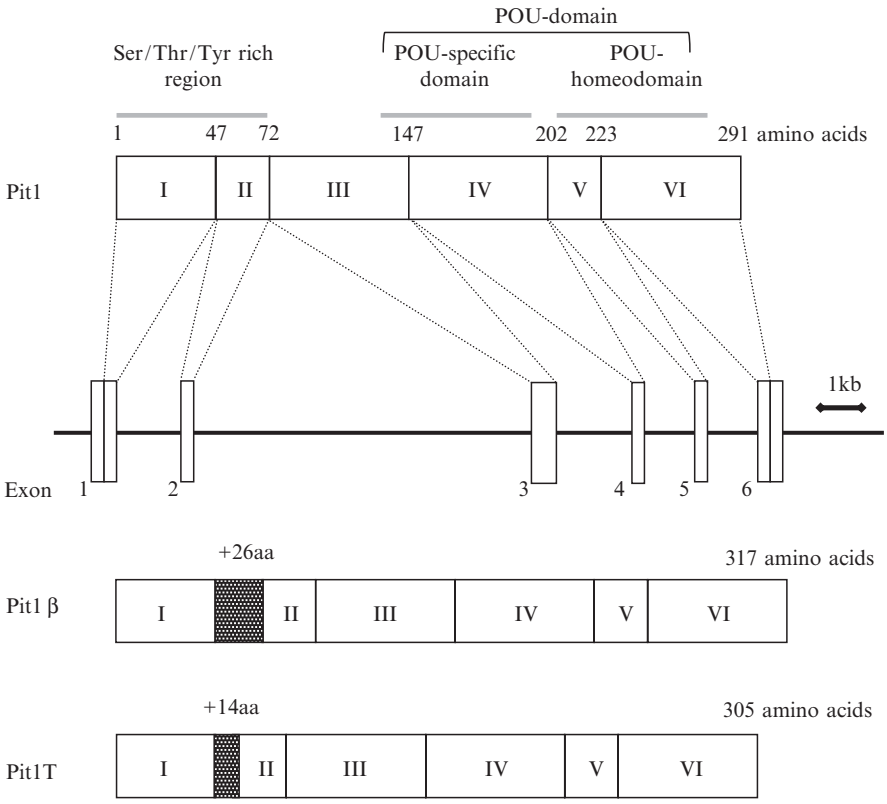
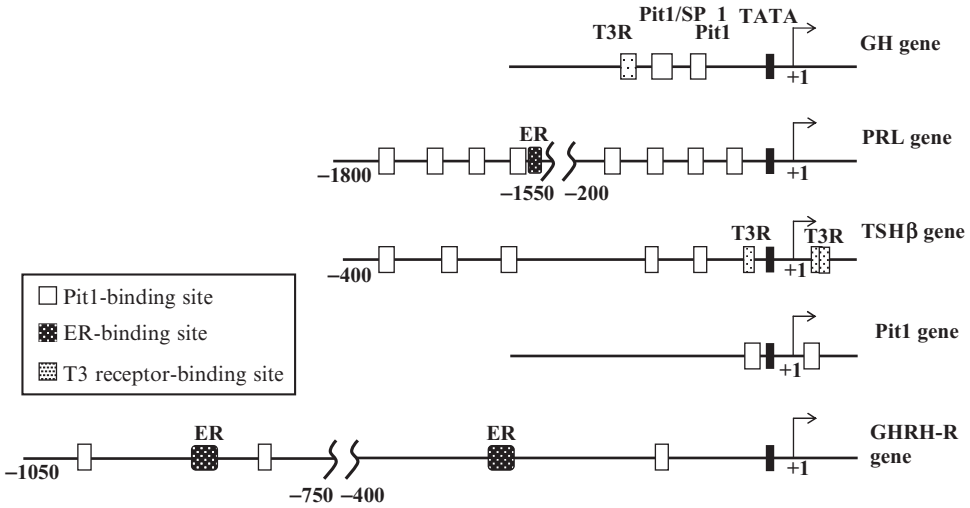


Fig. 4.5 Schemes for Pit1 molecule and its isoforms. Pit1 regulates the functional differentiation of the anterior pituitary cells to GH-, PRL-, and TSH-producing genes. Pit1 has six exons which code proteins of 291 amid acid sequence. Pit1 has two isoforms with insertions of 26 amino acids (Pit1β) and 14 amino acids (Pit1T)



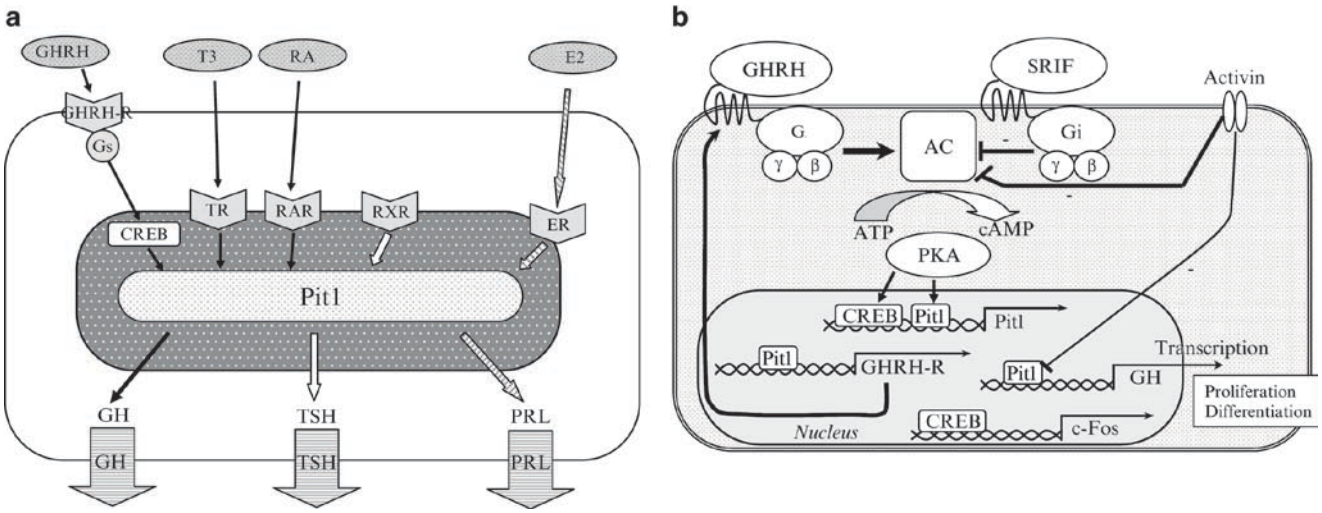


Fig. 4.6 Pit1-binding sites in GH, PRL, TSH, Pit1, and GHRH-R genes

Fig. 4.7 Pit1 function and GHRH-R signaling. (**a**) Hormone receptors to synergize with Pit1 activation. Various signals through respective receptors activate Pit1 and each hormone, GH, PRL and TSH, which is controlled by Pit1. (**b**) In GH-secreting cells, Pit1 functions GH transcription. In GHRH-binding GHRH-R and Pit1 activate GH secretion. Pit1 activates itself with PKA signaling

4.4.3 Cofactors Which Work Synergistically with Transcription Factors (Fig. 4.7a)

The cofactors may be classified as (a) those on the cell membrane, such as receptors for hypothalamic hormones, (b) those residing in the nuclei, such as nuclear receptors, (c) morphogenetic signaling, such as growth factor signals, and (d) novel signaling pathways.

4.4.3.1 Cell Membrane Receptors: Hypothalamic Factors Signals

The receptors for hypothalamic hormones are sevenfold transmembrane protein and include GHRH-R and GnRH-R as well as G-protein coupled receptors (GPCR). Pit1 and GHRH-R function synergistically in the production of GH in pituitary cells. GHRH-R has a Pit1 binding site in the upstream promoter region (Fig. 4.7b) [15]. GnRH-R stimulates the LH cells with GnRH binding and results in the activation of binding sites for SF1 in the upstream promoter region. Egr1 is activated by GnRH signaling, with Ptx1 and SF1 in LH-producing cells (Fig. 4.8). GnRH-R may play a role in the FSH cells, but detailed mechanisms still remain to be further clarified [16].

4.4.3.2 Nuclear Receptors

ER is a good example of this category which functions with Pit1 for the production of PRL. Dopamine receptor is another factor which negatively regulates the PRL production by binding to the promoter region of PRL gene. Retinoic acid receptor (RAR) is a DNA binding protein for GH production using the synergistic function with Pit1. Retinoid X receptor

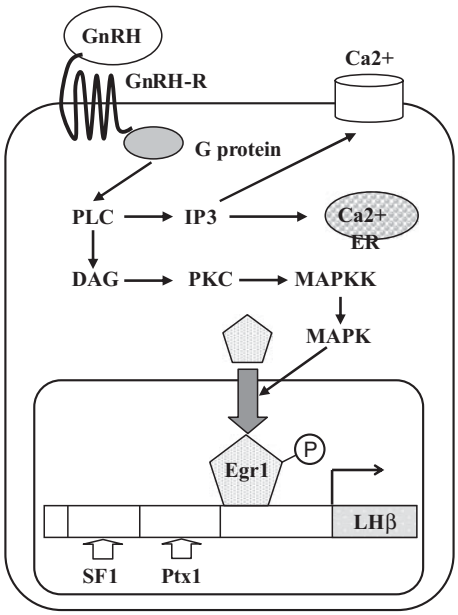


Fig. 4.8 Egr1 synergizes with Ptx1 and SF1 to rapidly increase LHβ gene expression. When GnRH binds to its receptor (GnRH-R), Egr1 is activated and it synergizes with Ptx1 and SF1. *DAG* diacyl glycerol, *IP3* Inositol trisphosphate, *PKC* protein kinase C, *MAPK* mitogen-activated protein kinase, *MAPKK* MAPK kinase

(RXR) is a factor which has been shown to play a role in the functional differentiation of TSH and/or GH with Pit1 binding (Fig. 4.9) [17].

4.4.3.3 Morphogenetic Signals

Morphogenetic signaling molecules within the ventral diencephalon and surrounding tissues, such as fibroblast growth factor 8 (FGF8), bone morphogenetic proteins 2 and 4 (BMP2, 4), sonic hedgehog (Shh), Wnt4, thyroid transcription factor (Ttf1/Nkx2.1) play critical roles in the early development of pituitary organogenesis (Fig. 4.10). These morphogenetic signaling molecules are synergized with several specific transcription factors for the early pituitary development, including Hesx1, Ptx1, Msx1, and Prop1. Analysis of these pituitary morphogenetic factors indicates that the ventral→dorsal and/or dorsal→ventral gradient signals constitute the functional positioning of hormone-producing cells in mouse pituitary development [18]. Wnt4 is also expressed and may play a role in the proliferation and survival of the PIT1 lineage pituitary adenoma cells [19].

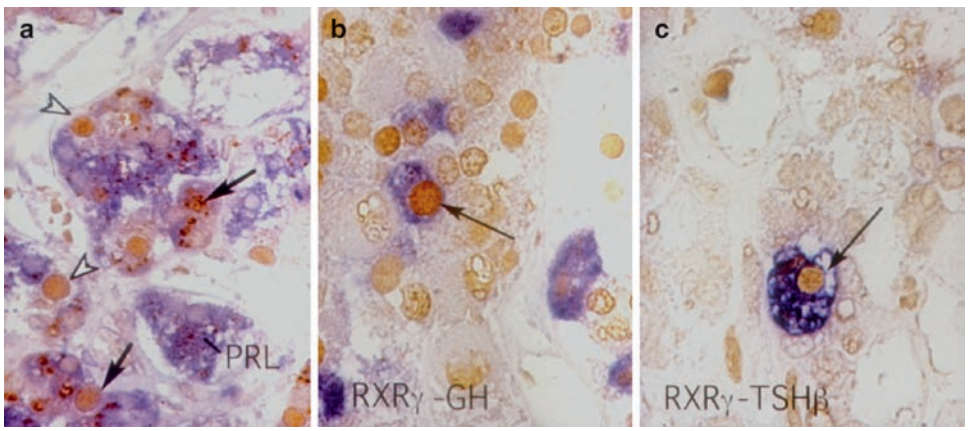


Fig. 4.9 Immunohistochemical co-localization of transcription factors (*brown*) and pituitary hormones in rat pituitary (*blue*). Each transcription factor was detected in the nuclei of anterior lobe. (**a**) ER was localized in the nuclei of PRL-secreting cells. RXR was localized in the GH (**b**) or TSH-secreting cells (**c**)

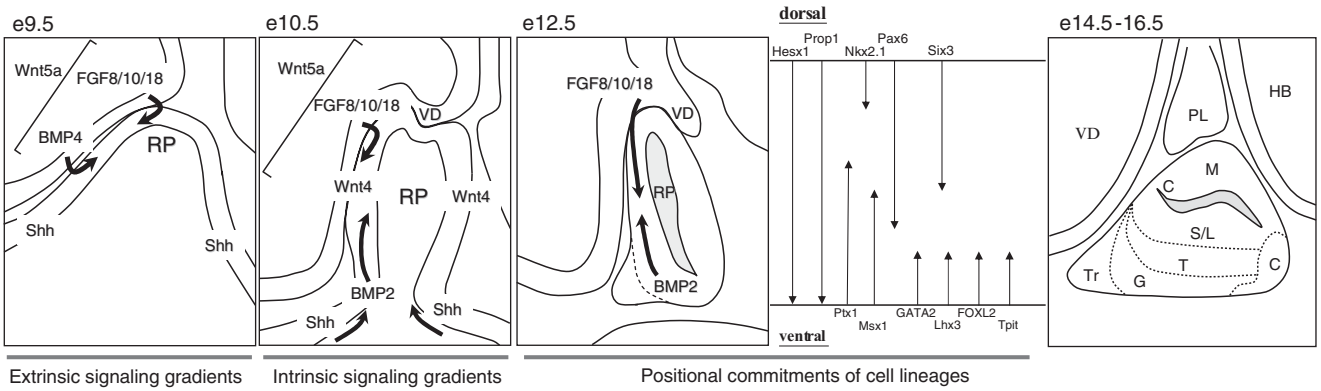


Figure 4.10 Morphogenetic signaling and transcriptional strategies in mouse during early pituitary development. By embryonic (e) 10.5, morphogenetic signaling from the ventral diencephalon including Wnt5a, FGF8/10/18, BMP4, and from the oral ectoderm including Shh, BMP2, Wnt4 are provided. By e14.5, a cascade of morphogenetic signaling molecules plays crucial roles in positional commitment of each cell lineages through the dorsal→ventral and/or the ventral→dorsal expressions. *RP* Rathke's pouch, *VD* ventral diencephalon, *PL* posterior lobe, *HB* hind brain, *M* melanotrope, *C* corticotrope, *S* somatotrope, *L* lactotrope, *T* thyrotrope, *G* gonadotrope, *Tr* thyrotrope in rostral tips (modified after Scully et al. 2002 [18])

4.4.3.4 Cell-to-Cell Interactions: Notch Signaling

Notch signaling controls the progenitor cell differentiation in many embryonic tissues. Notch signaling is mediated by the interaction between Notch receptor and its ligands, which are both cell-surface transmembrane proteins. Recently, it was reported that Notch signals, which regulate the maintenance of progenitor cells and the timing of their differentiation in various tissues and organs, play an important role in pituitary development. In early pituitary development, Notch1 signaling is required for maintaining the expression of Prop1, which is required for the GH-PRL-TSH lineage differentiation [20]. Prop1 is also required for normal Notch2 expression [21]. Pituitary precursor cells express the Notch signaling molecules, including the downstream effector gene Hes1. Hes1 is required for the survival and proliferation of Rathke's pouch precursor cells in early pituitary development, and is also necessary for melanotroph differentiation [22].

4.5 Loss of Function in Transcription Mechanisms and Disorders

Dwarf (*dw*) types include Pit1, Prop1, and Pit1 binding site of GHRH-R

The discovery of Pit1 was led by an analysis of Snell dwarf mouse (*dw*), which is known as dwarfism. The locus of Pit1 gene is clarified on mouse chromosome 16, consistent with *dw* mutation [23]. Jackson dwarf mouse (dw^J) is known as dwarfism as well as Snell dwarf mouse. Prophet of Pit1 (Prop1) has been isolated by a positional cloning in dwarf mouse (*df*) which is located on chromosome 11 [23]. The *df* mutation is the depletion of the GH, PRL, and TSH cells; it has been indicated that Prop1 is capable of an early enhancer of Pit1. In humans, it has been reported that the mutations of *PROP1* are responsible for the cause of combined pituitary hormone deficiency (CPHD), a disorder resulting from an impaired pituitary function and is characterized by an impaired production of GH and other pituitary hormones. The CPHD is presented with GH, PRL, TSH, and gonadotropin deficiency, recently a concomitant alteration of the corticotroph function has been described. Some mutations of *PROP1* in human 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 2bp GA deletion (296delGA) [24–29]. Lhx3 and Lhx4, LIM homeobox gene transcription factors, and Hesx1 are the factors which appear as earliest known factors for the Rathke's pouch. Hesx1 mutations in mice are of two types: Class I mutant showed the absence or substantial reduction of telencephalic vesicles, eyes, olfactory placodes, and frontonasal mass, and in contrast, Class II mutant exhibited less severe craniofacial dysplasia and frequently only one eye was affected. In both mutants, the anterior pituitary was small. Fibroblast growth factor 8 (FGF-8) expressed in the ventral diencephalon is reduced significantly in 8.5 days (Fig. 4.10). The analysis of Sonic hedgehog (Shh), 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 dorsal–ventral pattern of neural tube indicates that Shh is not expressed in Class I mutant and is normally expressed in Class II mutant. Nkx2.1 is absent in some mutants [30]. Pax6 is expressed similarly in mutants and wild-type. The mutants also indicate association of septo-optic dysplasia (SOD) (Fig. 4.10). In the screening of human SOD patients, some mutants have been discovered: C473T mutant results in the loss of Cac8I restriction site, A374G substitution in exon3, C509T mis-sense mutation, A541G mutant, and G18C mutant. In C509T, A541G and G18C mutants, GH deficiency is exhibited, and in G18C mutant, TSH and LH/FSH deficiencies are exhibited [31]. In animals with $Lhx3^{-/-}$ or $Lhx4^{-/-}$, the oral ectoderm invaginates to form the Rathke's pouch, and in animals with double knockout of both $Lhx3^{-/-}$ and $Lhx4^{-/-}$, the invagination occurred but resulted in a pouch rudiment [32]. 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 null Lhx3 mutation ($Lhx3^{-/-}$), GH, TSHβ subunit, αSU, LH, and Pit1 are deficient, but POMC is expressed not only in 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 null Lhx4 mutant, αSU, GH, TSHβ subunit, and Pit1 are present. However, only a few LHβ subunit positive cells and a few GnRH-R positive cells are present. Further, in these mutant animals, cell proliferation of the anterior lobe is affected and the anterior lobe is hypoplastic and the numbers of all five hormone-secreting cells are reduced. In humans, two mutations of Lhx3 have been discovered, one is homozygous A to G transition which results in a Y116C substitution and the other is a homozygous deletion of 23bp and the adjacent splice-donor site. In these cases, pituitary hormones are completely deficit except for POMC. The phenotype of this case is similar to the phenotype of the above-mentioned Lhx3 mutant in mice [33].

4.6 Gene Targeting Techniques to Study Molecular Mechanisms

Gene targeting, which includes the production of transgenic and knockout animals, is inevitable in studying the function of certain transcription factors or cofactors. GHRH-R is a receptor for GHRH which is produced and secreted at the

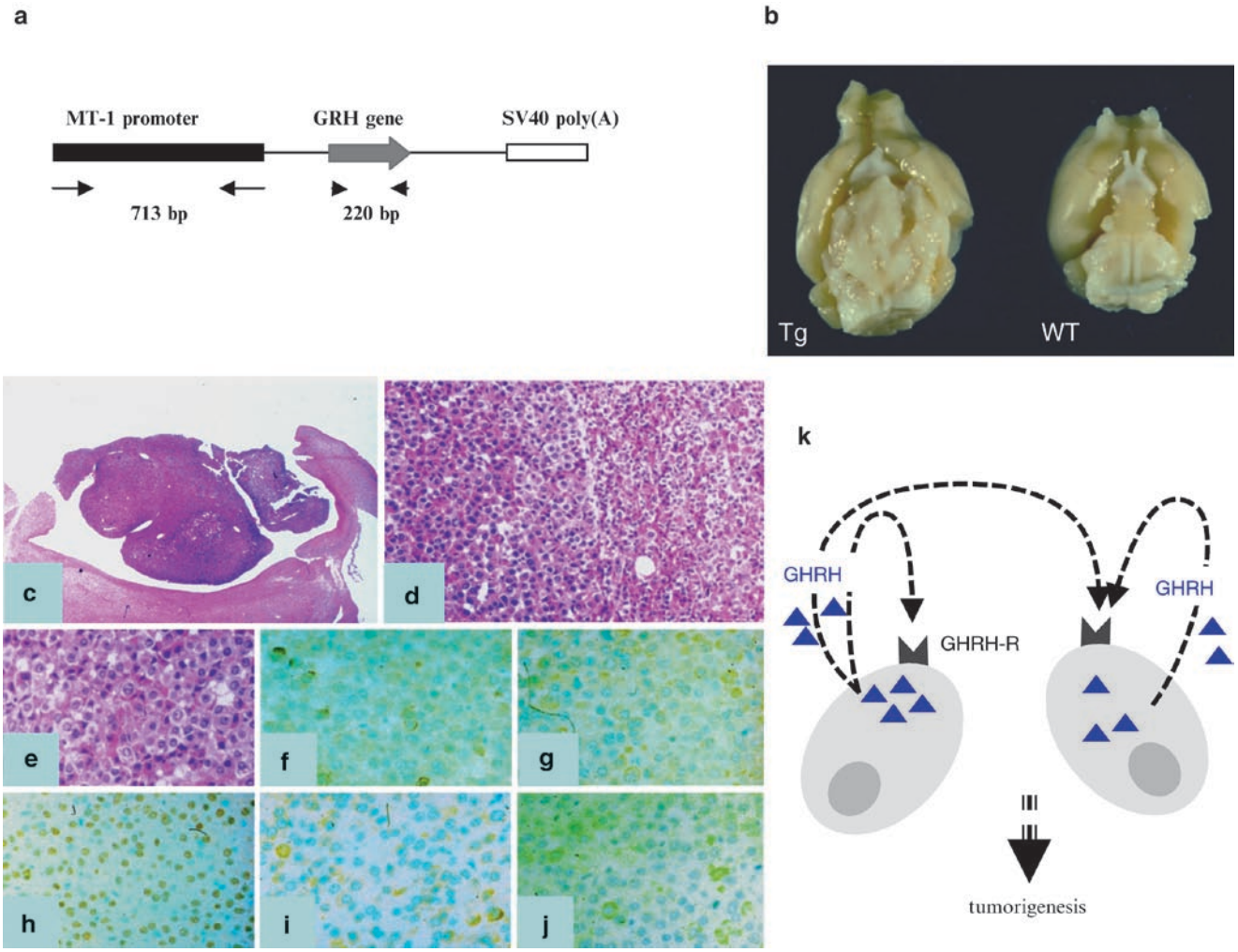


Fig. 4.11 *GHRH* transgenic mice (*GHRH*-Tg) develop pituitary adenomas. (**a**) Construct of *GHRH* transgene. The construct consisted of a 713 bp fragment of the mouse MT-1 promoter, containing elements responsible for metal induction and transcriptional initiation fused to the human *GHRH* gene. (**b**) *GHRH*-Tg had markedly enlarged and congested pituitary gland. The results of HE staining of the tumor are shown (**c**, **d**, **e**). The pituitary adenoma immunohistochemically express GHRH (**f**), GH (**g**), Pit1 (**h**), PRL (**i**) and TSH (**j**). (**k**) Scheme of mechanism for tumorigenesis in *GHRH*-Tg mouse

hypothalamus. GHRH-R is a GPCR with a molecular weight of 47 kDa. Pit1 binding sites are present in the upstreams of not only GH, PRL, and TSH genes, but also in the upstreams of GHRH-R and Pit1 genes (Fig. 4.6). *GHRH* transgenic mouse or rat has been successfully used as a model of GH-secreting pituitary tumors (Fig. 4.11). SF1 knockout mice in the anterior pituitary caused hypogonadotropic hypogonadism with sexual infantilism, sterility, and severe gonadal hypoplasia [34]. On the contrary, recent studies show that female mice with the constitutive *Prop1* transgene can develop GH, PRL, TSH, and/or gonadotropin-producing adenomas (Gn-omas) [35]. Prop1 plays the role of a tumorigenic factor as well as a differentiation factor to the Pit1-cell lineage (GH-PRL-TSH lineage) (Fig.4. 12).

4.7 Pituitary Stem and Progenitor Cells as Multipotent Cells

Tissue-specific stem cells have been uncovered in a growing number of organs by their molecular expression profile and their potential for self-renewal, multi-potent differentiation, and tissue regeneration. The nestin-expressing adult stem cells in the mature mouse anterior pituitary are terminally differentiated to six endocrine cell types of the pituitary gland [36]. A side population (SP) is also known as a technique for the selection of stem and/or progenitor cells). The adult pituitary contains SP cell, which have early embryonic characters [37].

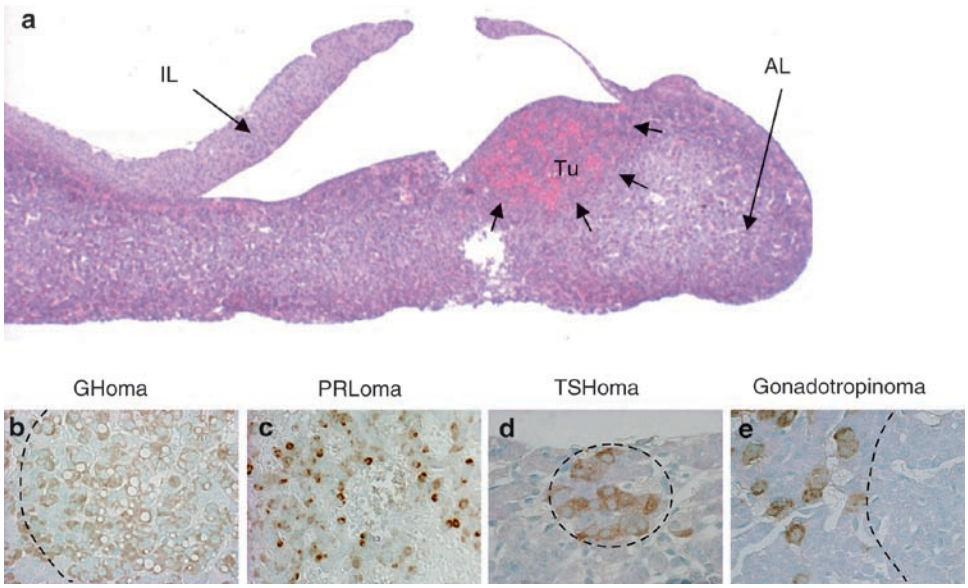
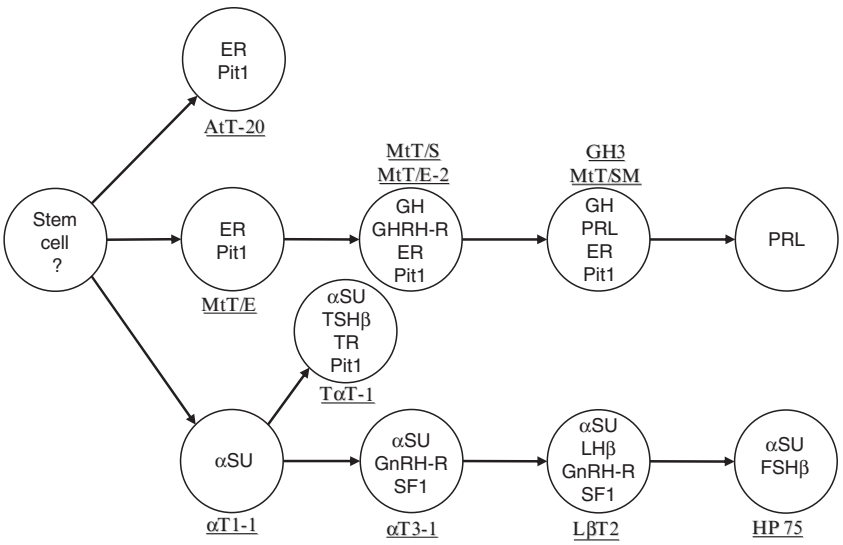


Fig 4.12 *Prop1* transgenic mice (*Prop1*-Tg) develop pituitary adenomas. (**a**) *Prop1*-Tg had markedly enlarged and congested the pituitary gland. The results of HE staining of the tumor are shown. Each pituitary adenomas immunohistochemically express GH (**b**), PRL (**c**), TSHβ (**d**), and LHβ (**e**). *AL* anterior lobe, *IL* intermediate lobe, *Tu* induced tumor region



Origin	Cell name	Species
GH	MtT/S	Rat
GH/PRL	GH3	Rat
	MtT/SM	Rat
TSH	TαT1	Mouse
LH/FSH	LβT2, LβT4	Mouse
αSU	αT3-1, αT1-1	Mouse
	HP75	Human
ACTH	AtT-20	Mouse
FS cell	TtT/GF	Mouse
	Tpit/F1	Mouse

Fig. 4.13 Pituitary cell lines. Each cell line produces individual pituitary hormone and/or hormone receptors

4.8 Cell Lines

Several cell lines have been established and used extensively for studying the detailed molecular mechanisms of differentiation. These cell lines include αT3-1 and LβT-2, as indicated in the table. These cell lines correspond to the developmental stages of various cell lineages (Fig. 4.13). Rat cell line MtT/S cells has been known as a GH-producing cell. GH3 and MtT/SM cells have been known as GH- and PRL-secreting cell lines [38]. MtT/E cell line is a non-functioning cell line derived from estrogen-induced mammotropic pituitary tumor (MtT) as well as MtT/S and MtT/SM cell lines [39]. MtT/E-2 cell line established from MtT/E cells is proliferated by estradiol stimulation, and the cells secrete GH [40]. These cells are in the cell line of GH- and PRL-secreting cells and express *Pit1* mRNA. Mellon et al. immortalized and established a few familiar cell lines (gonadotroph and thyrotroph cells), i.e. αT1-1, αT3-1, LβT2, TαT1. αT1-1 cell line which produces only αSU is an early progenitor cell [41, 42]. αT3-1 cells produce αSU and express SF1 and GnRH-R, LβT2 cells produce LHα, βSU and express SF1 and GnRH-R [43]. In αT3-1 cell lines, GnRH stimulates to increase GnRH-R and αSU. Cell-specific promotion of the function of αT3-1 cells lies in

the binding of SF1 and AP-1 as well as GnRH-R-activating sequence (GRAS) element. αT3-1 cells do possess activin and activin receptor [44]. TαT-1 cells produce αSU, TSHβ subunits and express Pit1. AtT-20 cells produce POMC and are expressed as Ptx1. Lloyd et al. established the HP-75 cell line from the human non-functioning plurihormonal adenoma, and are known to express FSHβ, LHβ and αSU [45]. The human pituitary cell lines including HP-75 are useful for future studies.

4.9 Specific Aspects of Human Pituitary Development and Pituitary Adenomas

As was briefly mentioned earlier, the human pituitary is unique in various aspects, such as in the fetal presence of intermediate lobe and the concomitant production of αSU in the GH-producing cells. "Invasive anterior cells" in the posterior lobe are also a unique counterpart of the distinct intermediate lobe in rodents (Figs. 4.1h and 4.2c).

Pituitary adenomas are infrequent intracranial tumors in human and are classified according to the function, i.e. GH-producing, PRL-producing, TSH-producing, ACTH-producing, gonadotropin-producing, and null cell adenomas. According to previous investigations, it has been clarified that the functional differentiation of the human pituitary adenomas, in general, are regulated by transcription factors and a combination with cofactors which regulate the normal pituitary cells as well as pituitary cell differentiation during fetus formation. The human pituitary adenomas are frequently multi-hormonal, but usually within a certain cell lineage. Occasionally, one adenoma produces multiple hormones belonging to different cell lineages, i.e. GH and ACTH. In these tumor cells, it has been found that the tumor cells contain PIT1 and NEUROD1 which do not collaborate in the normal pituitary cells [46–48]. This could be considered as an "aberrant" expression of transcription factors in neoplastic conditions.

4.10 Summary

This chapter emphasized the development and functional differentiation of anterior pituitary cells and adenoma cells covering the recently disclosed molecular mechanisms by various transcription factors and cofactors. In the pituitary cells, various molecular technologies have been demonstrated to analyze these molecular events including differentiation, proliferation, and tumorigenesis.

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Chapter 5
Recent Developments in the Molecular Biology of Pituitary Tumors

Ricardo V. Lloyd

5.1 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 has been implicated in familial and sporadic pituitary tumor development (Tables 5.1–5.3). However, many of the genetic abnormalities involved in the development of these tumors have not been uncovered as yet.

5.2 Familial Pituitary Tumors

Familial pituitary tumors constitute a small number of pituitary adenomas, probably accounting for less than 5% of these tumors [5–15]. These include tumors associated with multiple endocrine neoplasia 1 (MEN1) [7], tumors associated with Carney's Complex (CNC) [9–11] isolated somatotropinomas [13] and tumors associated with mutations in the aryl hypocarbon-interacting protein (AIP) gene [14, 15]. Most of these mutations are rare in sporadic pituitary tumors [5, 6].

The *MEN1* gene is inherited in an autosomal dominant manner. The MEN1 syndrome is caused by mutations in the *MEN1* gene on chromosome 11q13. This gene encodes the regulatory protein menin and is characterized by a series of endocrine functional and nonfunctional tumors as well as nonendocrine tumors (discussed in another chapter). Prolactin-producing tumors are most common in patients with MEN1 disease [5]. A recent report indicated that the gene encoding for $p27^{Kip1}$ (CDKN1B) was mutated and produced a MEN1-like syndrome in a rat model and in a human kindred [8].

Carney's complex (CNC) is a rare familial condition characterized by lentigines, myxomas, adrenal cortical nodular hyperplasia, and pituitary abnormalities [9, 10]. A mutation of PRKAR1A has been found in around 50% of cases [11]. Pituitary lesions are characterized by hypersecretion of PRL, GH and insulin-like growth factor I (IGF-1). Pituitary hyperplasia is frequently seen in these patients.

5.2.1 Isolated Familial Somatotrophinomas

This condition is defined as equal to or greater than two cases of acromegaly or gigantism in a family in the absence of MEN1 or CNC. More than 50 families with IFS including over 120 individuals have been reported [5]. It is characterized by slight role predominance and a young age of onset around 25 years compared to sporadic acromegaly and gigantism. The tumors are usually macroadenomas. Mutations in the AIP gene are now a potential cause of IFS. However, 50% of IFS families do not have mutations of AIP gene [5].

5.2.2 Familial Isolated Pituitary Adenomas

Approximately 50% of families with homogeneous acromegaly/IFS have AIP mutations [5]. These findings indicate that additional genetic abnormalities are associated with FIPA [5]. AIP is a member of the immunophilin family of proteins with three tetraicopeptide repeats. Some AIP mutations can lead to protein truncatum. AIP mutations are very uncommon in sporadic pituitary adenomas [5]. Patients with AIP mutations and pituitary tumors have a poorer response to therapy like many young patients with macroadenomas [5].

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Table 5.1 Genes/conditions involved in familial pituitary tumors

Gene/condition
Multiple endocrine neoplasia 1 (MEN1)
Carney's complex (PRKAR1A)
Isolated familial somatotrophinomas
Aryl hydrocarbon receptor-interacting protein (AIP)

Table 5.2 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 [22, 23]
Cyclin D	Cyclin D1 (CCDN1) gene shows allelic imbalances in some tumors. Cyclin D2 and D3 abnormalities may also be significant [24–27]
CREB	Constitutively activated cAMP-responsive nuclear transcription factor. CREB may facilitate GH cell transformation [18]
Ras	H-ras mutations detected in metastases from pituitary carcinoma [19, 20]

5.3 Oncogenes

5.3.1 Gsp Gene

The Gsp gene is an oncogene located on chromosome 20 and is a mutant form of the a-subunit of Gs transmembrane signaling protein. GSa is associated with a dominant activating mutation and has been observed in some growth hormone tumors ([3, 4] and Table 5.2). 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 [16, 17]. The constitutively activated cAMP-responsive nuclear transcription factor CREB might be promoted by GSa overexpression, and this may contribute to growth hormone tumor development [18].

5.3.2 Ras Gene

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 [19–21]. These findings suggest that H-ras may be a late event in human pituitary tumor development.

5.3.3 Pituitary Tumor Transforming Gene

The pituitary tumor transforming gene (PTTG) was recently cloned and characterized [22]. 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 tenfold increase in PTTG expression [23]. PTTG may be a new marker of invasiveness in secretory pituitary tumor [23].

5.3.4 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 that are essential to progression to the

Table 5.3 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 [7]
Rb	Loss of Rb expression seen in some animal pituitary tumors, but mutations uncommon in human pituitary tumors [30, 31]
p53	Overexpression of p53 protein common in pituitary carcinomas, but mutations infrequent [32, 33]
nm23	Reduced expression seen in invasive adenomas in one study [36]
ZAC	Expression reduced in null cell and gonadotroph tumors compared to normal pituitaries [37]
GADD45y	Expression reduced in nonfunctioning adenomas compared to normal pituitaries [38]
p16/CDKN2A	Hypermethylation frequent in some subtypes of pituitary tumors compared to normal pituitaries [47]
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 [48–57]
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 months. Status in humans not currently known [67, 68]
MEG3	Present in all anterior pituitary cells. Loss of MEG3 expression in nonfunctioning gonadotroph pituitary adenomas [69, 70]

next phase of the cell cycle. The D cyclins include D1, D2, and D3. These have been mapped to chromosomes llql3, 12p13, and 6p21, respectively. Cyclin D1 has been shown to be a protooncogene, and cyclin D2 may have similar functions [24, 25]. Cyclin D1 (CCND1) is often amplified in human tumors such as breast cancer. The cyclin D1 protein is coded for by the PRAD1-CCND1 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 [26]. 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 [26]. 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 [26]. 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 [27]. In a study of rodent pituitaries [28] and human pituitary tumors, very little cyclin Dl protein was expressed, while cyclin D3 was expressed by both functional and nonfunctional tumors [29]. Cyclin D2 was more commonly expressed by nonfunctional than functional tumors in a study of human pituitary adenomas [29].

5.4 Tumor Suppressor Genes

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

5.4.1 Retinoblastoma Susceptibility Gene

Mice with knockout of the retinoblastoma susceptibility (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 [30]. In one study, Simpson and co-workers found Rb expression in three growth hormone and 53 nonfunctional tumors, and 27% of growth hormone tumors and 4% of nonfunctional tumors did not express Rb proteins [31].

5.4.2 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 [32, 33]. Overexpression of p53 protein has been reported commonly in pituitary carcinomas [34, 35]. 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.

5.4.3 nm23

The *nm23* gene is located on chromosome 17q24q25. 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 [36]. 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.

5.4.4 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 promotes 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 [37].

GADD45rGADD45y, 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 *GADD45y* 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) [38]. The *GADD45y* gene was not expressed in most growth hormone or prolactin-secreting pituitary tumors [38]. This study showed that *GADD45y* expression was lost in the majority of human pituitary tumors. However, mutations of the *GADD45y* gene have not been reported in pituitary tumors.

5.4.5 p16/CDKN2

P16 or CDKN2A is a cell cycle protein and belongs to the INK family. This family also includes pI5/INK4B, pI8/INK4C, and pI9/1NK4D. These proteins have four ankyrin repeats and form complexes with CDK4 and/or CDK6. They also interact with D-type cyclins. One of the functional activities of the p16 protein is that they interact with the retinoblastoma protein [39]. The *p16* gene is located in chromosome 9p21 and is mutated in some tumors such as melanoma [40], supporting its role as a classical tumor suppressor gene. Woloschak et al. reported *p16* gene alterations in pituitary tumors. They observed that the gene was hypermethylated in some tumors compared to normal pituitary [41, 42]. 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 [43–46]. Frost et al. used a mouse pituitary cell line (ATT/20) and showed that transfection of the *pi6/CDKN2A* gene was associated with a reduction in cell proliferation, indicating that *pi6* could mediate cell growth arrest [47].

5.4.6 p27/Kip1

Another family of CDKI includes the Kip/Clp proteins, which include p27/Kip1 (p27), p21 /WAFlICip1(p21), and p57/Kip2 [p57]. These proteins inhibit kinase activities by pre-activated G1*cyclin E/CDK2 cyclin D-CDK4/6* and other cyclins. *p27* has been studied extensively [48]. Mice with *p27* knockout gene developed hyperplasia and tumors of the intermediate lobes of the pituitary [49–51]. Many studies in mice and humans have shown that *p27* is probably important in tumor development of pituitary as well as other tumors [52–59]. 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 48). Our laboratory first reported that ACTH tumors had the lowest levels of *p27*expression [57], and this has been confirmed by other laboratories [60].

Although *p27*is a putative tumor suppressor gene, the infrequency of mutations of this gene suggests 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 [58, 59]. 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 [61–63]. 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 [64]. The F-box protein SKP2, for example, is important in degradation of a phosphorylated *p27* [65] and JAB 1 is also important for *p27* degradation [66]. Additional studies are needed to examine the role of these genes in regulating p27 function and in pituitary tumor development.

5.4.7 p18/CDKN2C

This p18(CDKN2C) 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 [67]. Animals with both *p27* and *p18* knockouts developed massive pituitary tumors as well as hyperplasias and tumors of other endocrine tissues [67]. The pituitary tumors can lead to death of these mice by a few months of age [67]. Recent studies have shown that the p18 gene is methylated in mice with pituitary tumors and this was correlated with the lack of expression of the p18 protein [68].

5.4.8 MEG3

MEG3 is an imprinted gene expressed from the maternal allele. MEG3 expression was reported in normal gonadotrophs and was less in gonadotroph adenomas [67, 70]. Hypermethylation of the IG-DMR gene at the DLK1/MEG3 locus has also been found in gonadotroph adenomas [70], so this may be the mechanism of silencing in these tumors [70].

5.5 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 [71]. Alterations in various chromosomes including chromosomes 10, 11, and 13 have also been implicated by comparative genomic hybridization studies [72–81]. Comparative genomic hybridization studies have highlighted the complexity and the extensive alterations of chromosomal loci in pituitary tumors (Table 5.4) [72–81].

Table 5.4 Comparative genomic hybridization analyses of human pituitary adenomas

Reference	Tumor types	*N*	Abnormality (%)	Chromosomal abnormalities
[72]	Nonfunctional	23	74	Sex chromosome and 18 (34.7%); amplifications of 4q, 5q, 9p, 13q and 17q (10–30%)
[73]	PRL, GH, TSH, ACTH, nonfunctional	52	48	11, 7, X, 1, 8, 13, 5, 14, 2, 6, 9, 10, 12, 3, 18 (decreasing frequency); functional tumors > nonfunctioning tumors
[74]	GH	10	80	Gains 5, 9, 22q, 17p12 (20–50%); losses 13q, 18 (20–30%)
[75]	PRL, GH, nonfunctioning	12	–	Loss 13q most common (5 cases)
[76]	All types	75	45.3	Gains 4.9 times more frequent than lossesGains x (32%), 19 (16%), 12 (6.7%), 7 and 9 (6.7%) Loss 11 (5.3%), 13 and 10 (4%)
[77]	Nonfunctional (26) functional (12)	38		Gains 3, 7, 14, 6p and 20q
[78]	All types	13		Gains 9q, 16p, 17p, 19, 20qLoss 1p, 2q, 4, 5, 611q, 12q, 13q
[79]	All types	24		Gains 4q, 17, 19, 1p, 5, 20, 6q, 13q21, 16pLoss 1p, 11p, 17, 16p, 4, 10p, 12, 20, 22q, 13q, 9p
[80]	Pituitary carcinoma	4		Gain 5, 7p and 14q

N number of cases studied

Table 5.5 Studies of gene expression profiles in human pituitary tumors

Reference	Types of tumors analyzed	*N*	Genes of interest
[130]	All types	37	Folate receptor, (nonfunctioning adenomas), ornithine, decarboxylase (GH) C-mer proto-oncogene kinase C (ACTH)
[131]	All types	52	Metallothionein in ACTH
[132]	Nonfunctional	11	SFRP1, TLEZ, PITX2, NOTCH, DLK1
[133]	All types	20	LAPTM4B, BAG1, p18
[134]	Adenoma and carcinoma	4	ASCL1, ID2 TLE-4, LGALS3
[135]	Nonfunctional invasive and noninvasive adenomas	8	MMP-9
[136]	PRL	25	ADAMTS6, CRMP1, DCAMKL3, PTTG, ASK, CCNB1, AURKB, CENPE
[138]	HP75-TGF beta-treated nonfunctional adenoma cell line	Cell line and TGFβ treatment	RUNX1, WNT5B, SOX4, SMAD3
[139]	PRL	6	PIT-1, BAG1, NOTCH, E-cadherin

N number of cases studied

Additional cytogenetic studies have supported some of the CGH findings.

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 [82]. The *HMGA2* gene was found to be overexpressed in human prolactinomas and in other tumors [83].

5.6 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. Many studies in the literature documenting some of the genes involved in mouse pituitary tumors including null or knockout and transgenic models have been reported [84–87]. Some of these new genes such as folate receptor, ornithine decarboxylase, and C-met tyrosine kinase have been recently discovered to be overexpressed in pituitary tumors from cDNA array data analyses that are discussed in later sections.

5.6.1 Summary

1. Many genes influencing pituitary tumor development in humans are largely cell-type specific. The *Gsp* mutations are predominantly in GH tumors, with *pI6* hypermethylation observed mainly in nonfunctional tumors.
2. The genes influencing pituitary carcinoma development in humans are largely unknown [88]. Pituitary carcinomas are defined by the presence of metastatic disease, so aggressive pituitary adenomas would represent an intermediate stage in tumor progression in humans [88].

5.7 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 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

5.8 In situ Hybridization

Various steps are involved in ISH and preservation of nucleic acid is one of the important first steps in the procedure [89–91]. 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 (Fig. 5.1) or nonradioactive reporters, which are more commonly used today [92]. Nonradioactive detection is more rapid, but this is usually not as sensitive as a radioactive probe (Fig. 5.2).

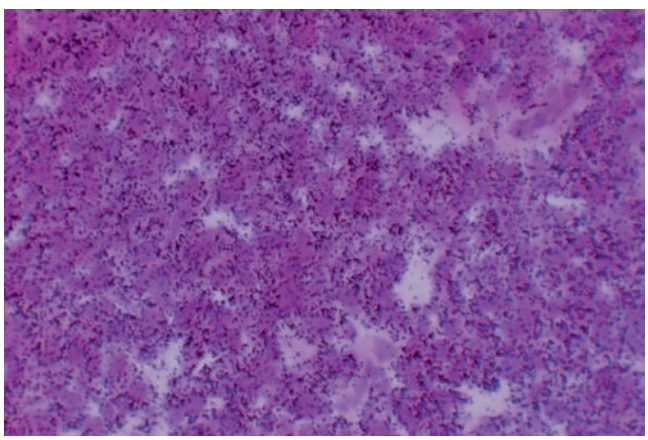

Fig. 5.1 In situ hybridization using a radioactive tritiated thymidine oligonucleotide probe demonstrating PRL mRNA expression in a prolactinoma. The black silver grains represent the positive signal

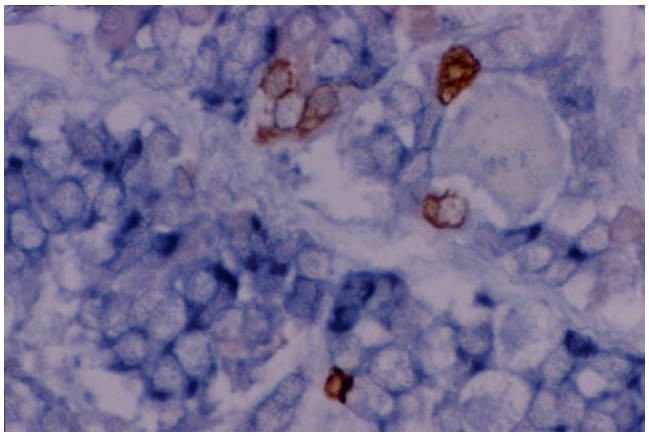

Fig. 5.2 In situ hybridization with nonradioactive chromogenic probes showing dual localization of PRL (blue color) and chromogranin A (brown color) in a prolactinoma. The signals were detected with alkaline phosphatase-NBT-BCIP for the PRL probe and biotin-DAB for the chromogranin A probe. Very few of the prolactinoma cells express chromogranin A in this tumor.

5.9 Hybridization Analysis in the Pituitary

Many studies have been done with ISH studies with human pituitaries (Figs. 5.1, 5.2). 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 [93]. 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 [94, 95]. Studies by Kovacs et al. [96] and Trouillas et al. [97] 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 [98] 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 [79]. In other studies of growth hormone and prolactin tumors, investigators [99] found that mRNA for prolactin increased during pregnancy [99]. This finding suggests that a transformation of cell types with the development of mammosomatotroph cells expressing both prolactin 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 a decreased cytoplasmic volume. A subpopulation of the larger prolactin cells did not show a decrease in prolactin mRNA [100].

ISH analysis of ACTH tumors has been done by various investigators [101]. 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 [102, 103]. Other studies have found that the silent ACTH tumors may have an abnormal mRNA [104]. Some investigators have used nonradioactive probes to detect POMC mRNA within pituitary tumors [105, 106]. Others have shown that in Nelson's syndrome, there was a greater detection of POMC mRNA compared to Cushing's disease, suggesting that other investigators have examined nonneoplastic pituitary for POMC gene expression and have shown that POMC mRNA can be detected in postmortem pituitaries up to 66 h after death [107]. Some investigators using quantitative ISH show that there was an increase in POMC mRNA in suicide victims compared to patients who had cardiac death [107, 108]. ISH analyses of gonadotroph tumors have been reported. The FSH beta and LH beta genes have been detected in gonadotroph, null cell, and oncocytic tumors [109–112]. This specific finding suggested a close relationship between gonadotroph and null cell tumors. Other studies of gonadotroph, nonfunctional pituitary tumors [99] show that one-third of the tumors expressed alpha 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 [110].

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 [111–114]. These studies have shown the utility of ISH in analysis of gene expression FISH has been used to analyze pituitary chromosomal abnormalities. These studies provide insight into the pathogenesis of pituitary tumors [81].

5.9.1 MicroRNA

MicroRNAs are small RNAs consisting usually of 19–23 bases which have important roles in regulating embryonic development [114, 115]. Recent studies have shown that they have critical roles in tumorigenesis where they regulate oncogenes and tumor suppressor genes [115, 116]. Specific microRNAs have been discovered in the pituitary gland and in pituitary tumors including microRNA-15a, 16-1 (Figs. 5.3a, b) and others [117–119]. Several of the microRNAs are involved in cell proliferation and apoptosis suggesting that deregulation could lead to pituitary tumorigenesis. Most microRNAs in the pituitary usually decrease in amounts during progression from normal anterior pituitary cells to adenomas. In situ hybridization has been used to localize specific microRNAs in normal pituitaries and pituitary adenomas in our laboratory (Lloyd et al., unpublished data).

5.10 In situ Polymerase Chain Reaction Analysis of Gene Products in Pituitary Tumors

Some studies have used reverse transcriptase-polymerase chain reaction (RT-PCR) for the analysis of gene expression in pituitary tumors [120–124]. In addition to the use of conventional RT-PCR for studies of pituitary tumors, investigators have used ISH and PCR *(*in situ PCR) to visualize the low abundant

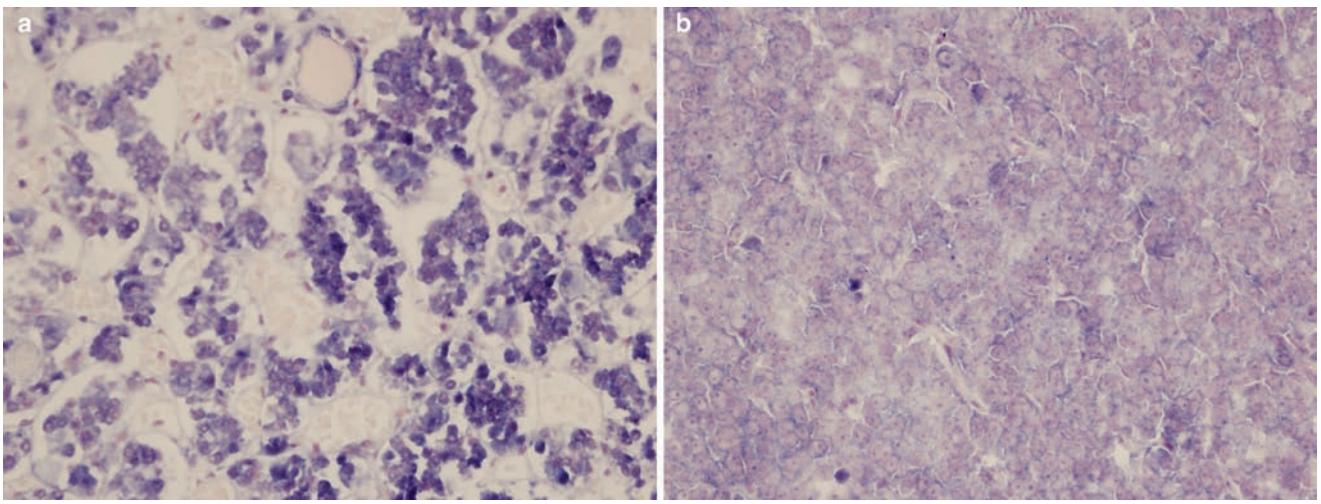

Fig. 5.3 **a** Localization of microRNA 15a in normal anterior pituitary showing strong positive staining with alkaline phosphatase NBT-BCIP. **b** Localization of microRNA 15a in a GH adenoma showing a weak positive signal compared to the normal anterior pituitary.

messages in cells [125–129]. 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.

5.10.1 DNA Microarray Studies

Large-scale analysis of eukaryotic gene expression using high throughput methods has led to new insights into pituitary tumor development and progression [130–139]. Evans and coworkers found several candidate genes in a study of 37 pituitaries [130]. Some genes were overexpressed in specific types of pituitary tumor [130]. Folate receptor was significantly overexpressed in nonfunctioning adenomas but was under expressed in PRL and GH adenomas. Many of these microarray studies have found different genes over expressed or under expressed in different tumor types. One gene that was noted in two independent sets of microarray studies was BAG1 expression by Morris et al. [133] and Evans et al. [139]. Hussaini et al. [135] observed that MMP-9 was expressed in aggressive nonfunctioning adenomas. This study was validated using a human nonfunctional cell line, HP75. In the only study including a pituitary carcinoma with metastatic disease, Ruebel et al. [138] found that galectin-3 (LGALS3), ASCL1, and TLE-4 were upregulated in the pituitary carcinomas [138]. Microarray studies have provided so much new data about gene regulation that it will require some time and extensive studies to determine the significance of many of these findings.

5.11 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 contribute to the discovery of new genes involved in the development of human pituitary tumors. More traditional molecular techniques such as cytogenetic studies, ISH RT-PCR and DNA microarrays have contributed an enormous amount of information about pituitary cell and tumor gene expression.

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Chapter 6
The Pineal Gland

Katherine M. Lindstrom and M. Beatriz S. Lopes

6.1 The Normal Pineal Gland

The human pineal is a small gland shaped like a pine cone and located posteriorly in the midline of the brain, at the quadrigeminal plate cistern. In the adult, the gland measures approximately 8 mm in its longest diameter and weighs about 100 mg. During development of the embryo, 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 remains 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 [3–5]. The pineal gland is one of the circumventricular organs of the brain; consequently, it lacks a blood-brain barrier [6].

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. 6.1). The main cellular population, which constitutes about 95% of the total gland, is a specialized neurosecretory cell named the 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 [7]. Pineocytes have features consistent with their neurosecretory nature exhibiting clear vesicles, dense-core granules, and synaptic organelles and ribbons [3]. Moreover, pineocytes may express neuronal-associated proteins, including neurofilament protein and synaptophysin (Fig. 6.1c).

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. 6.1d). 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.

Pineocytes are believed to represent modified neurons related 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 [8, 9]. 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 and rhodopsin [8, 10]. However, the role of human pineocytes in photoreception and phototransduction appears to be insignificant.

The pineal gland of all vertebrates, including that of the human being, appears to be able to secrete a number of peptide hormones [5, 8, 11]. Melatonin is the primary hormone of the pineal gland. The major physiological role of melatonin is its influence on sleep induction and circadian rhythmicity to changes in the photoperiodism, acting as a photoneurodendocrine transducer and biological pacemaker [4, 11, 12]. 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, and neurophysins I and II, pineal antigonadotropin, and a gonadotropin-releasing factor unique to the pineal [2, 5, 13]. The pineal gland appears to interact with various endocrine and neuroendocrine tissues to influence their metabolic activity. The pineal has an inhibitory effect upon gonadal function and is believed to affect the timing of puberty [13]. It also appears to play a role in immunosystem and cancer modulation [14–16]. An abundance of data has been accumulated in 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. More details of such discussion may be obtained from specialized books and review articles.

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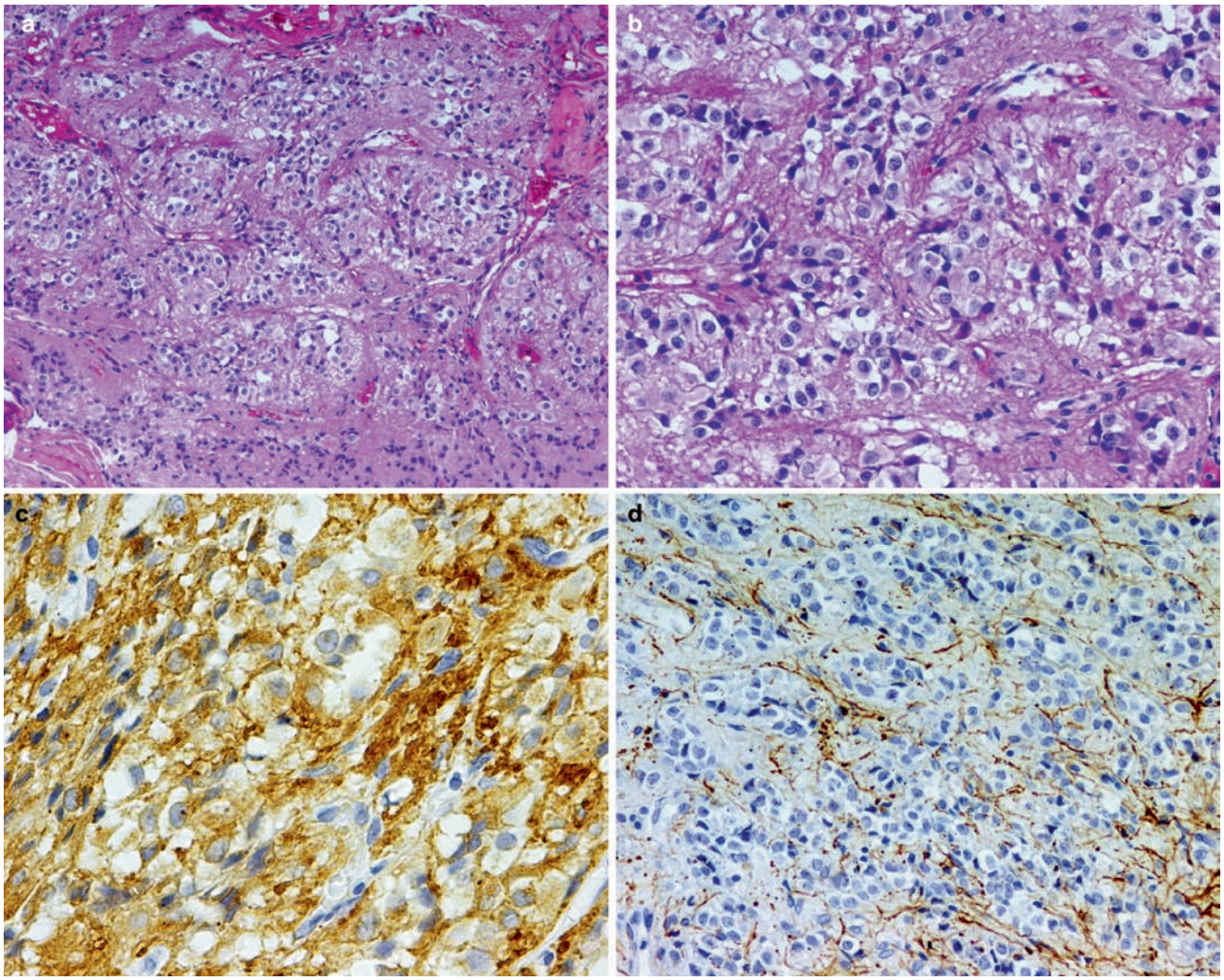

Fig. 6.1 Normal pineal gland – (**a**) The normal pineal gland has distinct lobular architecture with a prominent fibrovascular stroma. (**b**) The pineocytes are regularly arranged in the lobules displaying prominent cytoplasm and the typical neuroendocrine salt-and-pepper chromatin distribution. (**c**, **d**) Pineocytes are immunoreactive for neuroendocrine markers including synaptophysin (**c**), while stromal astrocytes are GFAP reactive (**d**). [(**a**, **b**) Hematoxylin & Eosin, (**a**) 100×, (**b**) 200×; (**c**) ABC immunoperoxidase for synaptophysin: 400×; D: ABC immunoperoxidase for GFAP: 200×; original magnifications]

6.2 Non-Neoplastic Lesions of the Pineal Gland

6.2.1 Pineal Cysts

Cysts of the pineal are the most common non-neoplastic lesions of the pineal gland. Small asymptomatic cysts are common incidental findings in neuroradiologic exams performed in cases of unrelated symptoms, and their incidence varies in the literature from 1.4% to as high as 10.8% [17, 18]. Cysts are also a frequent finding in autopsies with an incidence ranging from 25 to 40% [19].

The great majority of pineal cysts are asymptomatic. However, cysts 1.0 cm or larger may cause neurologic symptoms, most commonly non specific symptoms like headaches. Large symptomatic cysts have been reported to present as three main clinical syndromes [20]: (1) paroxysmal headache with gaze paresis; (2) chronic headache with signs of increased intracranial pressure including papilledema and hydrocephalus; and more rarely (3) pineal apoplexy with acute hydrocephalus [20–22].

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. 6.2). Occasional formation of Rosenthal fibers may be seen. The pineal parenchyma

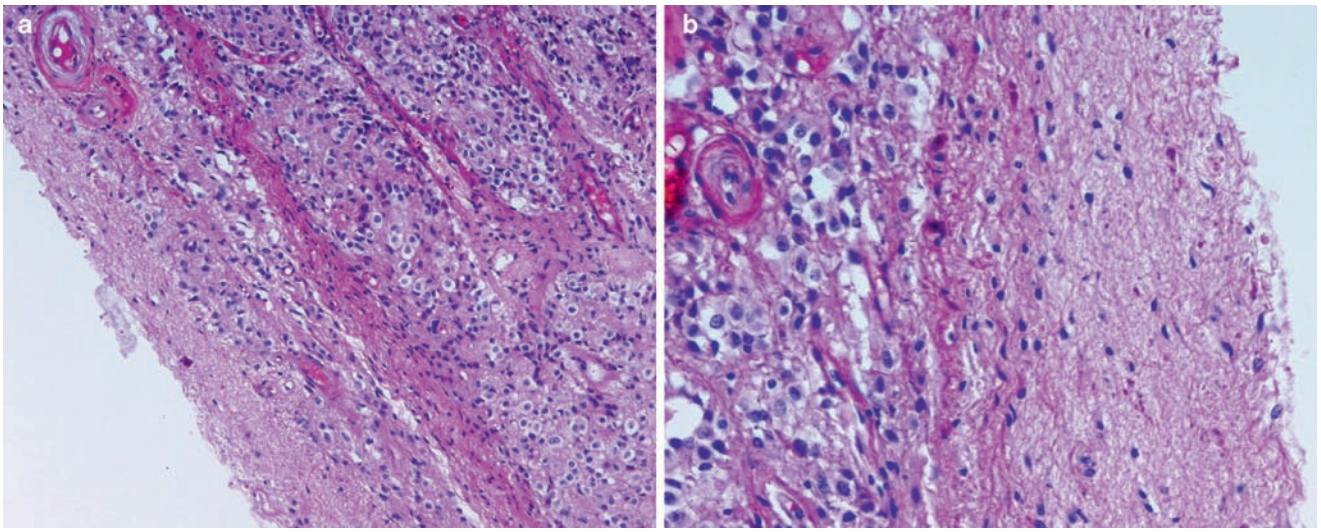

Fig. 6.2 Pineal Cyst – (**a**) Pineal cysts showing a hypocellular glial inner layer (*left*) compressing the normal pineal parenchyma (*right*). (**b**) A high-power magnification highlights the glial layer of a pineal cyst. [(**a**, **b**) Hematoxylin & Eosin, (**a**) 100×; (**b**) 400×, original magnifications]

surrounding the cyst may demonstrate variable degrees of distortion due to distention of the cyst. Recent and old hemorrhages are commonly observed particularly in cases of apoplexy [21]. The recognition of pineal cysts is critical as these lesions may be misdiagnosed as pineal tumors that require additional therapeutic intervention.

6.3 Tumors of the Pineal Region

Tumors of the pineal region represent approximately 1% of all brain tumors [23], though 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% due 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 (Table 6.1). Tumors arising from the pineocytes are designated as pineal parenchymal tumors (PPT) and have varying degrees of differentiation. The papillary tumor of the pineal region (PTPR) is a rare, recently described neuroepithelial tumor that is hypothesized to arise from specialized ependymal cells of the subcommisural organ [27]. Gliomas, most frequently astrocytomas, may arise from the supporting glial cells in a small proportion of cases. Meningiomas and non-meningothelial 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, is the germ cell tumors [23, 25, 28, 29].

6.4 Pineal Parenchymal Tumors

6.4.1 General Considerations

Pineal parenchymal tumors (PPT) comprise approximately 15–30% of the pineal region tumors [28, 30–36]. These intrinsic pineal tumors are divided by the World Health Organization (WHO) Classification of Tumors of the Nervous System [34] into three subtypes – pineoblastoma, pineocytoma, and PPT of intermediate differentiation (Table 6.2). 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, displaying 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 that 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 months in a large cohort study) [35], and low 5-year progression free survival (38% in another series) [37]. In contrast, pineocytomas commonly arise in older patients and tend to be more circumscribed lesions with no

Table 6.1 Classification and relative incidence of tumors of the pineal gland region

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

Table 6.2 WHO classification of pineal tumors

Pineocytoma (WHO grade II)
– Approximately 30% of PPT
– Mostly arising in adults (median age 35–47)
– Grossly well-circumscribed tumor
– Non-invasive of surrounding brain structures
– Histologically well-differentiated with characteristic pineocytomatous rosettes
Pineoblastoma (WHO grade IV)
– Approximately 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 neuroepithelial cells with high mitotic activity
Pineal parenchymal tumor of intermediate differentiation (WHO grade II or III)
– Intermediate grade of PPT with less distinctive clinical and histological features
– This category includes tumors with either histologic features intermediate between the two previous tumors or tumors that have mixed areas of both tumors
– This variant may metastasize by CSF pathways
Papillary tumor of the pineal region (WHO grade II or III)
– Rare neuroepithelial tumor of the pineal region
– Slight female predominance
– Solid and papillary tumor, histologically similar to an ependymoma or choroid plexus tumor
– Locally recurrent

propensity for (CSF) seeding. Pineocytomas are considered Grade II neoplasms by the WHO [34], and survival times are longer than pineoblastomas (5-year survival of 67%) [32]. The intermediate PPT appears to have transitional behavior according to the presence or absence of a major pineoblastomatous element [32, 34]. These tumors are designated as Grade II–III by the WHO classification [34]. Their potential for local infiltration and CSF dissemination is significant, and their clinical behavior appears to be more aggressive than pineocytomas [32].

6.4.2 Histological Types

6.4.2.1 Pineoblastomas

Pineoblastomas are the most primitive of the PPTs (Fig. 6.3). These tumors tend to be more common than the pineocytomas, accounting for up to 18% of the pineal region tumors [28, 33, 38, 39] and for 50–75% of the PPTs [32–34]. They most commonly occur in the first two decades of life [32–34] with a slight male predominance [7, 34]. Neuroimaging studies demonstrate multilobulated masses with heterogeneous contrast enhancement and poorly defined margins with the adjacent structures [40]. Pineoblastomas are highly invasive tumors that tend to disseminate thought the CFS pathways (Fig. 6.3d). Extraneural metastases after surgery have been described in two cases; one case in a thoracic vertebra and the other in the sacrum [41].

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. 6.3a–c). The cells are arranged in patternless sheets; however, neuroblastic Homer Wright rosettes may be present. Increased mitotic activity is frequently present (Fig. 6.3b), and areas of necrosis are common. Endothelial proliferation is present in great number of the cases [36].

Pineoblastomas may show a degree of photosensory differentiation with the formation of occasional photosensory Flexner-Wintersteiner rosettes [7, 42, 43]. These tumors may also rarely exhibit differentiation to mesenchymal tissues, including striated muscle and cartilage, and melanotic pigmentation [44, 45]. 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 [46–48]. The immunophenotype of pineoblastomas are consistent with both neuronal and neurosecretory differentiation (Fig. 6.3e) (see below). Pineoblastomas are classified as grade IV tumors by the WHO classification [34].

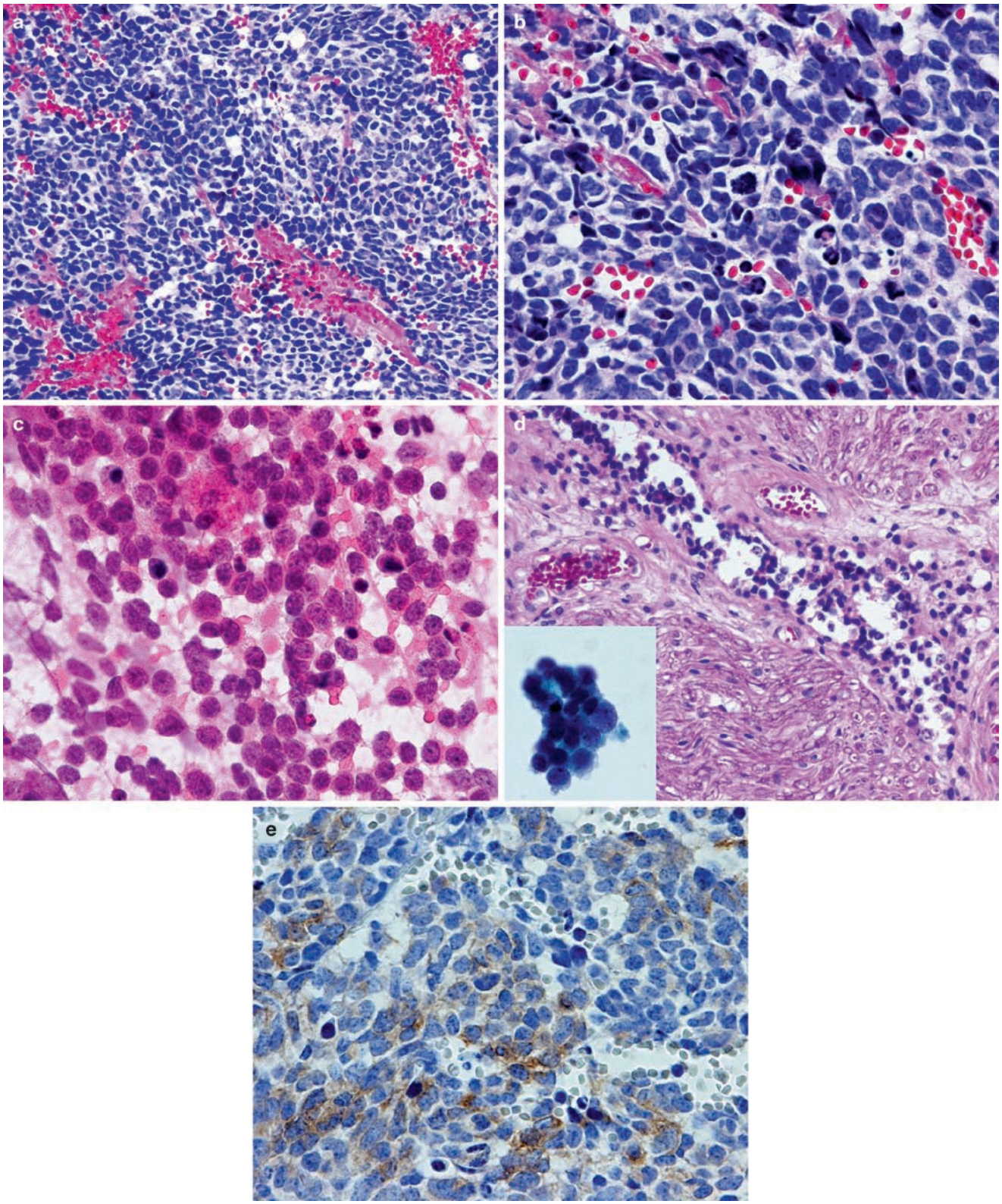

Fig. 6.3 Pineoblastoma – (**a**) Pineoblatomas are hypercellular tumors composed of small, undifferentiated cells with hyperchromatic nuclei. (**b**) Mitotic figures may be conspicuous (*center*), and foci of necrosis are commonly present. (**c**) Smear preparation of a pineoblastoma accentuates the 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**) Hematoxylin & Eosin, (**a**) 100×; (**b**) 400×; (**c**) 400×; (**d**) 200×; (**d**) *inset*: Papanicolau, 400×; (**e**) ABC immunoperoxidase for synaptophysin, 400×; original magnifications]

6.4.2.2 Pineocytomas

Pineocytomas account for about 7–30% of the PPTs (Fig. 6.4). The tumors may occur at any age, but commonly arise in young adults (median age of 35–47 years) with equal gender distribution [23, 32, 34, 36, 49]. In contrast to pineoblastomas, pineocytomas are well delineated, slightly lobulated tumors with clear margins with adjacent brain structures by neuroimaging [40], consistent with their small tendency for 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. 6.4a, b). Frequently, the cells are arranged in large pineocytomatous rosettes [50] (Fig. 6.4b). 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 [7]. 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" [36]. However, the presence of pleomorphic cells does not have any prognostic significance [36]. Mitotic figures are rare. Focal necrosis may occasionally be seen.

The neuronal and secretory nature of pineocytomas is demonstrated by immunohistochemical studies (see 6.4.3). 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 synapse-like structures [46]. Neurosensory cilia (9 + 0) and centrioles are also present [47, 48, 51–54].

6.4.2.3 Pineal Parenchymal Tumor of Intermediate Differentiation

A significant number of PPTs do not fit exactly into the categories of pineoblastoma or pineocytoma and exhibit behavior intermediary between these two distinct tumors. This fact has led to the introduction of the category of PPT of intermediate differentiation, which, in one series of cases, accounted for 56% of the PPTs [36]. Definite grading criteria have not been established for this diagnosis, but it appears to lie on a spectrum of differentiation with higher cellularity and fewer lobules and rosettes than a pineocytoma but lacking the small cell, primitive appearance of a pineoblastoma. Moderate mitotic activity and areas of necrosis may also be present [34]. It is still controversial as to exactly where to draw the line between these three entities [55]. One study has suggested using mitotic activity and immunoreactivity for neurofilament protein to divide PPTs of intermediate differentiation into two separate grades [36]. However, this has not been adopted by the WHO, and they are currently considered grade II or III.

6.4.3 Neurosecretory Differentiation of PPT

Immunohistochemical studies have confirmed the neurosecretory differentiation of PPTs. Primarily, these tumors express neuronal-associated proteins including neurofilament

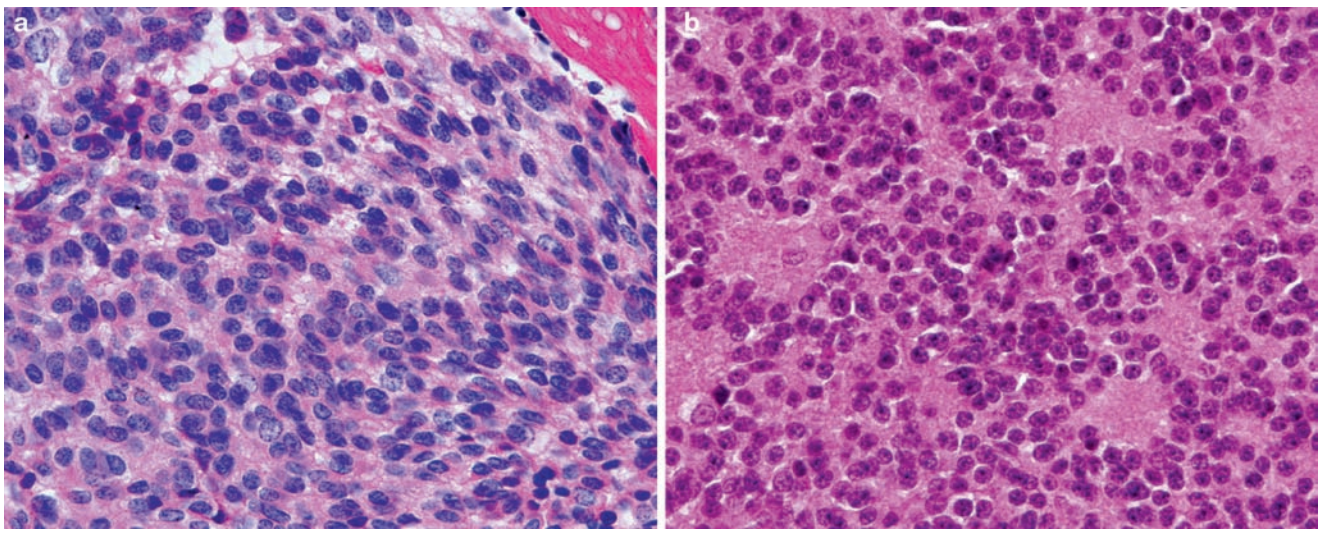

Fig. 6.4 Pineocytoma – (**a**, **b**) Pineocytomas are composed of uniform population of differentiated, "mature" cells arranged in solid sheet (**a**) and/or in the characteristic "pineocytomatous" rosette (**b**). Pineocytoma displaying elongate, delicate neuritic processes within the center of the rosettes [(**a**, **b**) Hematoxylin & Eosin, 200×]

proteins, synaptophysin, neuronal-associated class III β-tubulin, microtubule-associated Tau, and alpha-synuclein [36, 56–59] (Fig. 6.3e) in variable levels depending on the degree of tumor differentiation. While pineocytomas show intense reactivity for the majority of these markers, pineoblastomas may show reactivity to a few markers and with lower intensity [36]. Similarly, neuroendocrine markers such as chromogranin and neuron-specific enolase (NSE) are seen in the majority of the tumors but proportional to the degree of differentiation of the tumor [36]. GFAP and S-100 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 [8]. The photosensory-associated protein retinal S-antigen (S-Ag), a photoreceptor membrane-associated soluble protein, has been reported in pineoblastomas [10], pineocytomas [10, 36, 60, 61], and intermediate PPTs [36]. Interphotoreceptor retinoid-binding protein (IRBP), an interphotoreceptor matrix protein that functions in retinoid transport between the photoreceptor cells and the retinal pigment epithelium, is another photosensory marker that has been demonstrated in a PPT of intermediate differentiation [57].

6.4.4 Markers of Prognostic Value

Very few studies have evaluated morphological features of PPTs as prognostic and/or predictive factors [35, 36]. A series of 66 cases of PPTs analyzed a number of histological and immunohistochemical features and their role in patient survival and tumor recurrence [36]. These authors have found that the number of mitoses (>6/10 hpf) 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 higher survival rate. This group has suggested using these features as grading criteria for PPTs.

6.4.5 Molecular and Cytogenetic Profile of PPT

The mechanisms involved in the tumorigenesis of PPT remain not completely understood. Molecular and cytogenetic data on PPT are sparse, probably due to the rarity of these tumors (see ref. [61] for a comprehensive review). Cytogenetic studies performed on single cases of pineocytomas and pineoblastomas have found few distinctive chromosomal abnormalities in these tumors. Pineocytomas have shown losses of all or part of chromosomes 11 and 22 and deletions in the distal 12q region [62, 63], while in pineoblastomas, the most common abnormality appears to be deletion of chromosome 11q [64, 65]. A somatic truncated mutation of the *hSNF5* gene on chromosome 22q11 has been demonstrated in one case of pineoblastoma raising the possibility of a linkage with other CNS primitive neuroectodermal tumors [66]. Comparative genomic hybridization (CGH) has been performed in a series of nine PPTs [67]. Although no chromosomal gains or losses were found in pineocytomas (3 cases), the most frequent DNA copy number changes in pineoblastomas (3 cases) and PPT of intermediate differentiation (3 cases) were gains of 12q, 4q, 5p, and 5q, and losses of 22, 9q, and 16q. A more recent study used microarray analysis to determine differential gene expression among PPTs [68]. They identified certain genes, which are overexpressed in higher grade tumors, and hypothesized that they may be useful in the grading of PPTs of intermediate differentiation. 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.

Patients with germline *Rb* mutations are predisposed to develop pineoblastomas in association with retinoblastoma (the so-called trilateral retinoblastoma). However, loss of genetic material on chromosome 13q is not a prominent finding in either sporadic or familial pineoblastomas [61].

6.5 Papillary Tumor of the Pineal Region

New to the 2007 WHO classification [34] is the papillary tumor of the pineal region, a rare neuroepithelial tumor of which roughly 50 cases have been reported to date [27, 69–76]. This entity was first described in 2003 by Jouvet et al. [27] and in the past these tumors were likely diagnosed as a variety of things including pineal parenchymal tumors, ependymomas, papillary meningiomas, and choroid plexus tumors. A subsequent larger study has shown that this tumor occurs predominantly in adults, with a median age of 29 years (range 5–66 years), and has a slight female predominance [69]. Patients often present with a headache due to hydrocephalus. On imaging, these tumors are well-defined masses often having a cystic component. On MRI, they are isointense on T1-weighted images and hyperintense on T2-weighted images with contrast enhancement [70].

Microscopically these tumors are composed of a solid proliferation of cells with a significant papillary component in which vascular cores are covered with layers of tumor cells (Fig. 6.5). The cells are generally large, cuboidal, or columnar, with well-defined cell membranes and round to oval nuclei with stippled chromatin. Many cells are vacuolated containing amylase-resistant, PAS positive material.

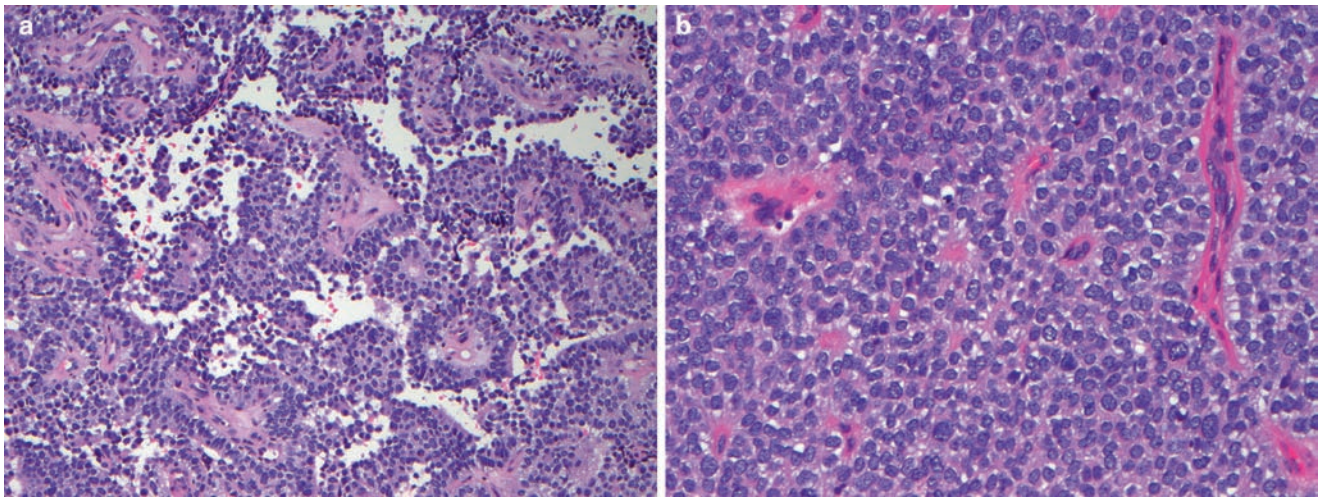

Fig. 6.5 Papillary Tumor of the Pineal Region – (**a**) These tumors have a significant papillary component with vascular cores covered by layers of tumor cells which are large, cuboidal, or columnar, with round to oval nuclei with stippled chromatin. (**b**) A solid proliferation of cells is also present which may exhibit perivascular pseudorosettes, true rosettes, and tubes. [(**a**, **b**) Hematoxylin & Eosin, (**a**) 200× (**b**) 400×]

Perivascular pseudorosettes, true rosettes, and tubes are present in varying degrees. Areas of necrosis are virtually always present. Mitoses are moderate in numbers, with a range of 0–10 mitoses per 10 high power fields [69]. Vascular proliferation is not seen, but vessels are often hyalinized. In order to distinguish a PTPR from other primary papillary tumors of the CNS and metastatic papillary carcinomas additional studies may be necessary.

Immunohistochemical stains help confirm this tumor's neuroepithelial differentiation. Analyses have shown these tumors to be uniformly positive for broad spectrum cytokeratin, CK18, S100, MAP-2, NCAM, NSE, and vimentin [69, 71]. Synaptophysin and chromogranin A may be weakly positive. Similar to ependymomas, EMA positivity has been seen in ring and dot-like patterns [27, 69, 72]. However, it is usually weak, and, unlike ependymomas, these tumors are negative for GFAP, except occasionally in perivascular cells. PTRTs are also negative for the choroid plexus tumor markers Kir7.1 and stanniocalcin-1 [71]. The Ki-67 proliferation index is variable, with a roughly even distribution of cases having indices less than 5%, 5–10%, and greater than 10% [69].

Electron microscopy demonstrates both ependymal and secretory differentiation, based upon the presence of microvilli, zipper-like junctions, dense core granules, numerous mitochondria, ependymal-like processes, and rough endoplasmic reticulum filled with secretory material [27, 74]. On the basis of the immunohistochemical profile and the ultrastructural analysis, an origin from specialized ependymal cells of the subcommisural organ has been postulated [27]. A recent microarray analysis of gene expression on two PTPRs gives support to this theory [68].

Other genetic studies on these tumors are limited, but one study of five PTPRs using comparative genomic hybridization demonstrated a mixture of chromosomal imbalances. The most common were losses on chromosomes 10 and 22q and gains on chromosomes 4, 8, 9, and 12 [71].

Because of the rarity of this tumor entity, studies of prognosis are difficult. However, one follow-up of 29 cases calculated a 5-year overall survival of 73% and a 5-year progression-free survival of 27%. Tumors tend to recur locally with rare spinal metastases reported. Gross total resection was the only factor that was associated with survival and recurrence, but it did not meet statistical significance. Increased mitotic activity did not affect survival or recurrence rates [69].

6.6 Germ Cell Tumors

6.6.1 General Considerations

Germ cell tumors (GCT) comprise the largest group of neoplasms in the pineal region, representing nearly 50% of tumors [23, 25, 28, 29, 33, 35, 77]. 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 being diagnosed between 11 and 20 years of age [23, 33, 34, 78]. A sex predilection is seen regardless of the type of GCT, with a male to female ratio of 14:1 in one series [77]. The association of GCTs of the pineal region with GCTs in other locations in the brain, particularly the suprasellar region, ranges from 2 to 12% of the reported cases [79].

GCTs include five basic categories: germinoma, embryonal carcinoma, choriocarcinoma, yolk sac (endodermal sinus) tumor, and teratoma [34]. Mixed GCTs, i.e. tumors with two or more histological types, may also occur in high percentages [32]. 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 GCTs appear to be similar to tumors of gonadal or extragonadal location, and they are believed to arise from the neoplastic transformation of primordial germ cells. It is hypothesized that during embryo development most of the primordial germ cells migrate to the urogenital ridges that give origin to 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 central nervous system at the diencephalo-pineal region [80]. 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. Additionally, the clinical presentation of the majority of the germ cell tumors coincides with the changes occurring in this region at the time of puberty [78, 79]. For an up to date discussion on the possible histogenetic theories of the central nervous system GCT, the reader should refer to Rosenblum et al. [81].

6.6.2 Histological Types

6.6.2.1 Germinoma

Germinomas are the most frequent GCTs involving the pineal region, accounting for 50–80% of such tumors [24–26, 77]. 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 a 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. 6.6). The tumor cells are arranged in large lobules separated by delicate fibrovascular septa. The latter contain lymphoid or lympho-plasmacellular infiltrates with large numbers of T-lymphocytes and activated macrophages [82]. A granulomatous reaction with aggregates of epithelioid histiocytes may also be present [83].

Immunohistochemistry of germinomas shows strong reactivity for c-kit and OCT4 [84, 85] and less likely placental alkaline phosphatase (PLAP) [81] (Fig. 6.6b, Table 6.3). C-kit and OCT4 appear more reliable than PLAP when applied to less differentiated tumors that may manifest PLAP antigenicity [84, 85]. Human chorionic gonadotropin (β-HCG)

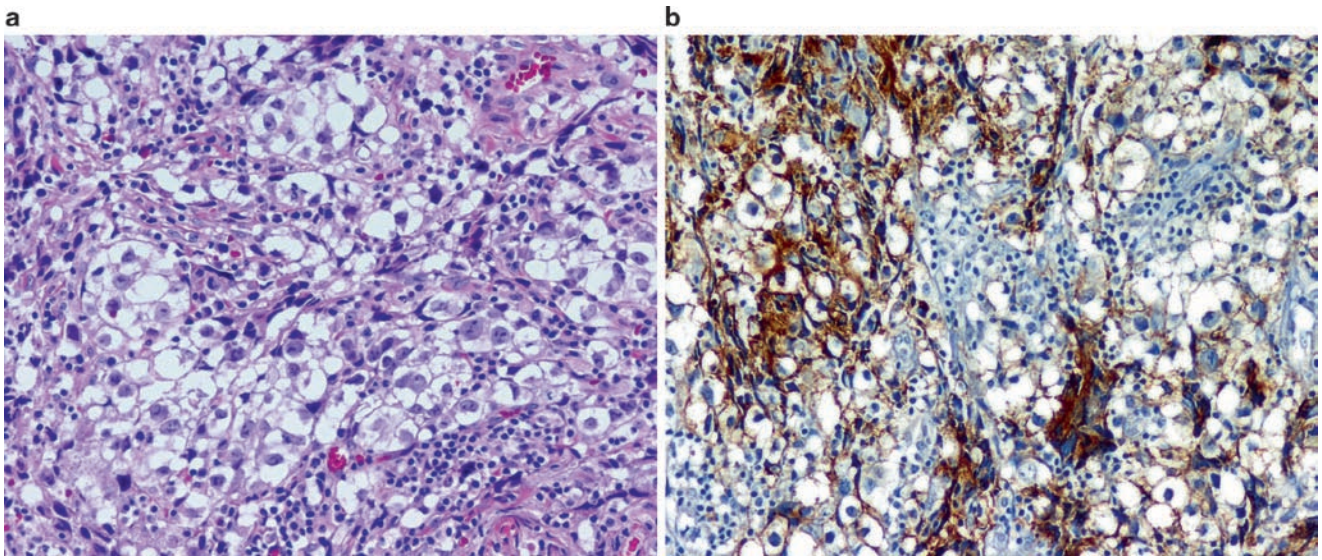

Fig. 6.6 Germinoma – (**a**) Germinomas are composed by dual populations of large, pleomorphic polygonal cells and small lymphocytes infiltrating the fibrovascular stroma. (**b**) The large tumor cells are distinctly positive for placental alkaline phosphatase (PLAP). [(**a**) Hematoxylin & Eosin, 200×; (**b**) ABC immunoperoxidase for PLAP, 200×; original magnifications]

may be sporadically positive in typical tumor cells [86]; however, β-HCG staining may also indicate the presence of syncytiotrophoblast-like cells within the tumor [86]. Germinomas with syncytiotrophoblastic elements may behave differently than ordinary germinomas. There are reports on higher recurrence rates [87] and shorter survival times [88] in β-HCG-producing germinomas than in pure germinomas. However, as compared to other GCTs, germinomas have a more favorable prognosis [77].

6.6.2.2 Embryonal Carcinoma

Embryonal carcinomas are very primitive GCTs, second only to germinomas. They are rare as pure tumors, constituting only about 5% of all intracranial germ cell tumors [78]. More frequently, embryonal carcinomas are a component of a mixed GCT, 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 epithelial-like arrangements including papillae and gland-like structures. High mitotic activity and extensive areas of necrosis are commonly present.

The immunohistochemical profile of embryonal carcinomas is remarkable for diffuse cytokeratin reactivity which distinguishes this tumor from germinomas (Table 6.3). PLAP and OCT4 are present in the majority of the tumors [81, 83], whereas AFP and β-HCG may be focally seen in a minority of cases [86, 89]. CD30 is expressed in these tumors [81], but c-kit expression is not seen in embryonal carcinomas [84].

6.6.2.3 Yolk Sac Tumor (Endodermal Sinus Tumor)

Yolk sac tumors are highly malignant GCTs. In the majority of the cases survival rates do not exceed 14 months [90]. These tumors represent about 7% of intracranial germ cell tumors [33, 78], and their preferential location is the pineal region [83, 90]. However, about 50% of the pineal cases are in fact mixed GCTs containing various proportions of yolk sac tumor and other germ cell elements [90].

Yolk sac tumors are characterized by the presence of pseudoglandular structures formed by endodermal cells arranged in a myxoid matrix [7, 81, 91]. The tumor cells are often clear, cuboidal to columnar, epithelial-like cells arranged in sheets, cords, and variable tubular formations to true papillary structures (Fig. 6.7). The presence of distinct perivascular epithelial-lined structures, named "Schiller-Duval bodies," is typical in these tumors. Another common feature is the presence of eosinophilic, PAS-positive and diastase-resistant hyaline droplets located in the cytoplasm or free in the stroma [86].

Yolk sac tumors are characteristically immunoreactive for AFP in both the epithelial cellular component (Fig. 6.7b) and the hyaline globules (Table 6.3). In fact, elevated AFP in CSF and/or blood alone appears to be strongly suggestive of an endodermal sinus tumor [92].

6.6.2.4 Choriocarcinoma

Pure choriocarcinomas are extremely rare [29, 93], but the pineal region appears to be the preferential site for these tumors, accounting for about 75% of the intracranial cases [7]. 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 [78].

The tumor is characterized by extra-embryonic 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 [84, 93]. PLAP and cytokeratin may also be positive in these tumors [83, 89] (Table 6.3).

Table 6.3 Immunohistochemistry of germ cell tumors

Tumor	PLAP	AFP	β-HCG	Cytokeratins	c-kit (CD 117)	OCT4	CD30
Germinoma	+	−	−	±	+	+	−
Embryonal carcinoma	+	±	±	+	−	+	+
Yolk sac tumor	±	+	−	+	−	−	−
Choriocarcinoma	±	±	+	+	−	−	−

+ = Immunoreactive
± = May be focally immunoreactive
− = Immunonegative

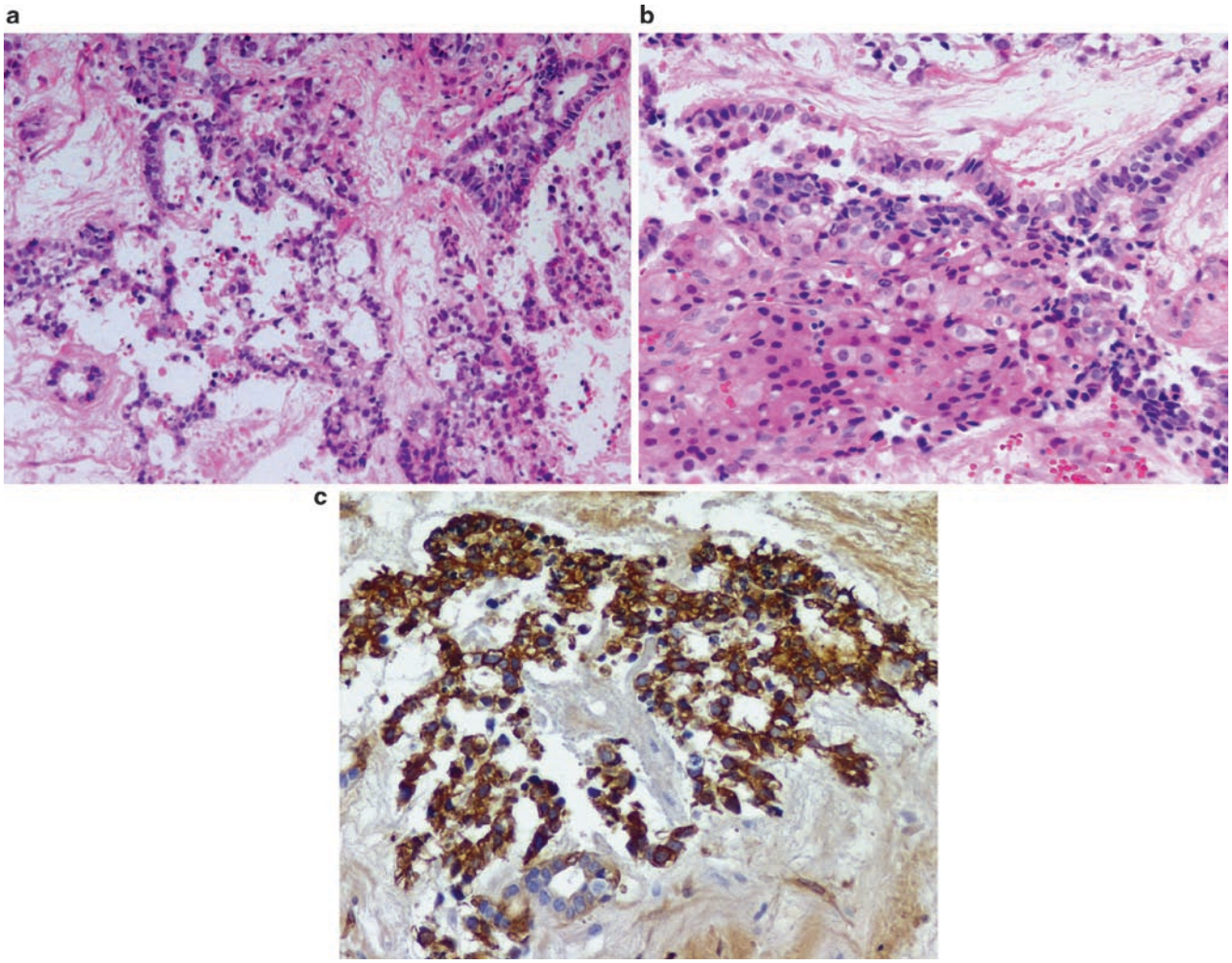

Fig. 6.7 Yolk sac tumor – (**a**, **b**) An example of yolk sac tumor showing papillary structures (**a**) admixed with a more diffuse cellular arrangement (**b**). (**c**) Alpha-fetoprotein is characteristically reactive in these tumors. [(**a**, **b**) Hematoxylin & Eosin, (**a**) 100×; (**b**) 200×; (**c**) ABC immunoperoxidase for alpha-fetoprotein, 200×; original magnifications]

6.6.2.5 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 about 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 [7, 25, 94]. The tumors usually occur in younger patients than germinomas, and in a recent epidemiologic survey, teratomas had the lowest mean age at diagnosis of 11 years [77]. Similar to germinomas, there is a marked male predominance.

Teratomas are often large, well-circumscribed masses that tend to firmly adhere to the adjacent parenchymal structures. The tumors are generally multicystic and composed of a mixture of 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 that the immature teratomas. The majority of these 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, e.g. skin with adnexa, cartilage and bone, adipose tissue, bundles of striated and smooth muscle, glioneuronal tissue with choroid plexus, retina with pigmented ocular epithelium, etc.

Immature teratomas are the most common form of intracranial teratomas [29, 33]. 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 [95]. Immature teratomas tend to disseminate to the CSF pathways more frequently [29, 96, 97], and recurrence and death rates are higher than those of mature teratomas [29, 95]. 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 [98].

Teratomas with malignant transformation are tumors in which there is a conventional "malignant" component of somatic type, for example carcinomas and sarcomas, alongside the regular mature or immature teratomatous elements [31]. Rhabdomyosarcoma or undifferententiated sarcomas are most commonly seen [81]. These tumors are less frequent that 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."

Malignant germ cell elements, particularly germinoma and embryonal carcinoma, are often seen in combination with mature and immature teratomas [5, 81]. The mixture of germinoma and teratoma has been estimated to occur in about one-fifth of teratomas [99]. In these mixed germ cell tumors, the classification of "teratoma with germinoma" or "teratoma with embryonal carcinoma" appears to be more appropriate than the old expression "teratocarcinoma."

6.6.3 Cytogenetics and Molecular Genetics

Cytogenetic analyses of intracranial GCTs 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 [100–105]. The presence of isochromosome 12p [i(12p)], the most common karyotypic abnormality of gonadal GCTs [106], has been demonstrated in several cases [102, 104, 105–108] suggesting analogous genetic pathways in the formation of gonadal and intracranial GCT. An additional X chromosome and additional chromosome 21 were also commonly seen in pineal GCTs. This may be associated with the increased risk of GCT which is observed in patients with Klinefelter's syndrome and Down syndrome [99]. A subset of germinomas exhibit mutations involving c-kit [84].

6.6.4 Prognosis and Predictive Factors

The survival for any malignant pineal germ-cell tumor is estimated in 79.4% for 2-year relative survival and 73.7% for 5-year relative survival [77]. However, the histological type of GCT seems to be the most important prognostic factor for patient's survival. Comparatively, pure germinomas have longer survival and lower recurrence rates than any other GCTs. The 5-year survival rate for pure germinomas ranges from 80 to 95%, while 10-year survival may reach 91% [72, 77, 87, 88]. Malignant GCTs including choriocarcinoma, embryonal carcinoma, yolk sac tumor, and mixed germ cell tumor have the lowest survival rates with 5-year survival around 44% [32, 77, 88]. Teratoma survival rates vary according to their degree of differentiation. While mature teratomas have 5-year survival rates as high as 93%, immature teratomas, and teratomas with malignant transformation have a lower 5-year survival rate of 75% [88].

While treatment is often variable for GCTs, improved survival rates have been reported with regimens that incorporate radiation therapy with or without surgical resection [72, 77].

6.7 Tumors of the Neuroglia

Gliomas involving the pineal region are less common than PPTs, with an incidence ranging from 15 to 25% [33, 35]. Astrocytomas are the most common gliomas involving the pineal and are hypothesized to derive from the supporting glial cells of the normal pineal gland. Other gliomas may also involve the pineal region, but do 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 due to the close proximity of the gland to both ependyma and choroid plexus of the posterior roof of the third ventricle [109]. In a European cohort of 281 pineal region tumors gliomas represented 25% of the cases, these included astrocytomas (52/72), ependymomas (13/72), oligodendrogliomas and mixed oligoastrocytomas (7/72) [35].

All varieties of astrocytomas have been reported as involving the pineal, including the well delineated and relatively benign pilocytic astrocytoma (WHO grade I) and diffusely infiltrating astrocytomas (grade II–IV) [28, 110–114] (Fig. 6.8). Pilocytic astrocytomas of the pineal region behave

in a similar benign fashion as the ones arising in other locations of the CNS. Because of the circumscription of the tumors, they frequently are totally resected resulting in a favorable prognosis for the patients [28, 33]. Diffusely infiltrating astrocytomas are associated with a poorer prognosis. Most of the cases preclude total surgical resection, with management of the tumor by diagnostic biopsy followed by radiotherapy [33]. 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 in this localization than low-grade astrocytomas [28, 33, 113, 114].

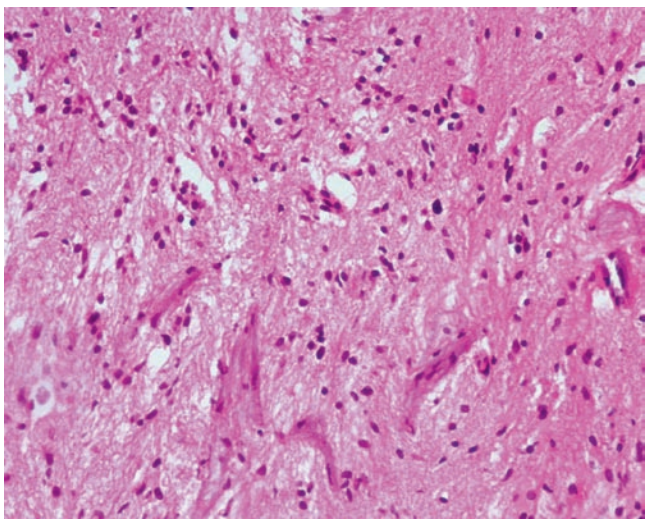

Fig. 6.8 An example of diffuse astrocytoma (WHO grade II) of the pineal region showing low-grade features, similar to astrocytomas of other regions of the CNS. [Hematoxylin & Eosin, 200×]

6.8 Tumors of the Meninges

6.8.1 Meningiomas

Meningiomas of the pineal region are rare and comprise about 5–10% of pineal tumors [115–118]. They may involve the pineal region by two mechanisms. Some 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 [119]. Alternatively, meningiomas may arise from the junction of the falx and tentorium and secondarily invade the posterior third ventricle and the pineal gland.

Pineal meningiomas appear to occur in a younger population than tumors in other locations [117, 119]. In the three largest series of meningiomas arising in the pineal region [117, 118, 120] the mean ages were 28, 41, and 49 years and lower than that reported for meningiomas in general (peak 50–59) [34]. Similar to meningiomas of other CNS regions, there is a female predominance. All histological subtypes of meningiomas have been described in the pineal region [119] (Fig. 6.9a).

6.8.2 Other Mesenchymal Tumors

Other mesenchymal tumors involving the meninges have been reported to occur at this location including hemangiopericytomas [121, 122] (Fig. 6.9b) and lipomas [97].

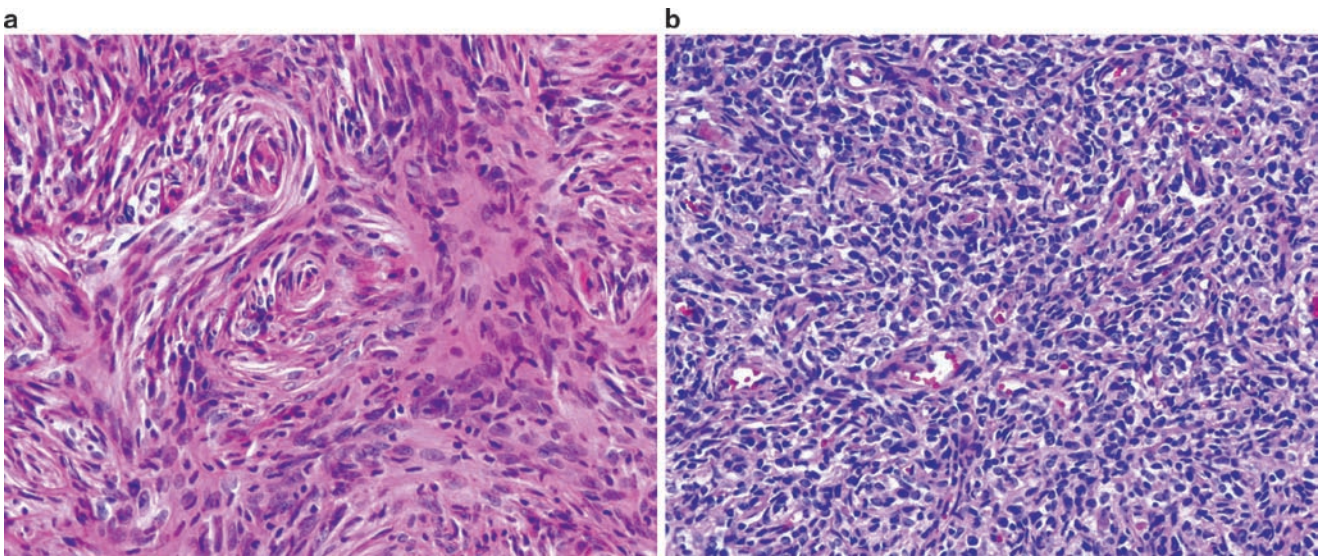

Fig. 6.9 Meningeal Tumors – (**a**) A meningothelial meningioma arising in the pineal gland of a 36-year-old female. (**b**) An example of hemangiopericytoma involving the pineal region. [(**a**, **b**) Hematoxylin & Eosin, (**a**) 200×; (**b**) 100×; original magnifications]

6.9 Miscellaneous Tumors

The pineal gland may rarely harbor metastatic carcinomas. There have been few reports of lymphoma involving the pineal gland [123] and a single case of primary Langerhans cell histiocytosis [124].

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Chapter 7
The Parathyroid

H. Rubén Harach

7.1 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 parathyroid glands by showing that their removal accounted for the fatal seizures in dogs following thyroidectomy. Von Recklinghausen described in 1891 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 acromegalic patient showing a pituitary tumor and enlarged parathyroid glands at autopsy now interpreted as MEN1. In 1915, Schlagenhaufer proposed that the bone disease was the result rather than the cause of the parathyroid tumors. In 1925, Mandl removed in Vienna 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 PTH being accomplished independently by Hanson in 1924 and Collip in 1925. A tentative diagnosis of the 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 units of parathyroid extract a day 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 the 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 PTHrP) secreted by the tumor. In 1958, Cope and collaborators recognized another type of primary chief cell hyperplasia [1–3]. This historical approach merges 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 [1, 4]. In practice, the management of parathyroid disease, especially hyperparathyroidism, requires a multi-disciplinary approach that includes the participation of the endocrinologist, surgeon, and pathologist. This chapter will mainly deal with the morphological aspects of parathyroid disease and differential diagnoses from the pathologist's viewpoint; the molecular aspects of the parathyroid glands are thoroughly approached in another chapter.

7.2 The Normal Parathyroid

7.2.1 Embryology and Anatomy

The parathyroid glands derive from the endodermal third and fouth 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-week human embryos. The third pharyngeal pouch wall extends caudally beyond parathyroid III (inferior) and forms thymus III from its ventral part (Fig. 7.1). In the 6-week-old embryo, the parathyroid III

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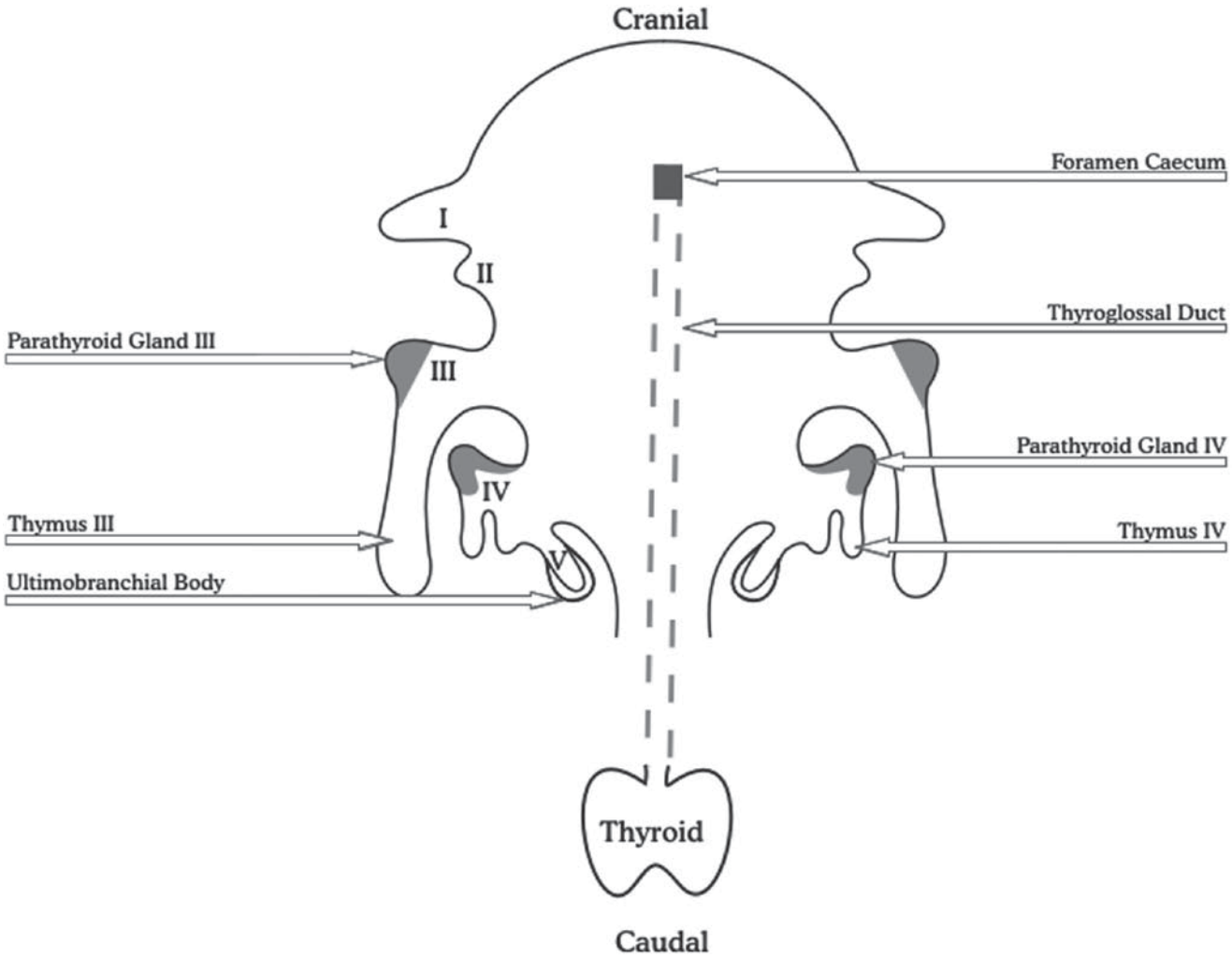

Fig. 7.1 Schematic embryologic derivation of the parathyroid glands and thymus

and thymus III complex is completely separated from the pharynx. Subsequently, 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-week-old embryo. If this separation fails, the parathyroid III will migrate to the lower neck within the thymus or into 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 together with thymus IV (accessory thymus) and the ultimobranchial body (Fig. 7.1). 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 the thymus IV tissue locates outside the thyroid [5, 6]. Several genes seem to be implicated in human parathyroid gland development [7]. The fetal parathyroid chief cell starts to show PTH immunoreactivity from 10 weeks of gestation [8], before acquiring its more classic adult-type morphological features by early childhood [8–10].

Most humans have four parathyroid glands. They are tan to red-brown in color and are flattened, oval, or bean-shaped measuring about 6 mm in length, 3–4 mm in width, and 1–2 mm in thickness. Occasionally, the parathyroids show an elongated or lobulated configuration [11–13]. The combined weight at 6 months of age is less than 10 mg, at 1 year 20 mg, at 5 years 30–40 mg, at 10 years is up to 60 mg, and in adults is about 140–160 mg. The upper limit weight of individual glands is generally considered as 40 mg at autopsy [12, 14–17]. In a recent study, the mean weight of normal surgically removed individual parathyroids was found to be considerably higher (62.4 mg), with 44% of the glands weighing 60 mg or more [16]. In about 80% of the cases, the superior and inferior pair of parathyroid glands shows a symmetrical anatomical position. The superior parathyroids are usually located in connective tissue at the cricothyroid junction

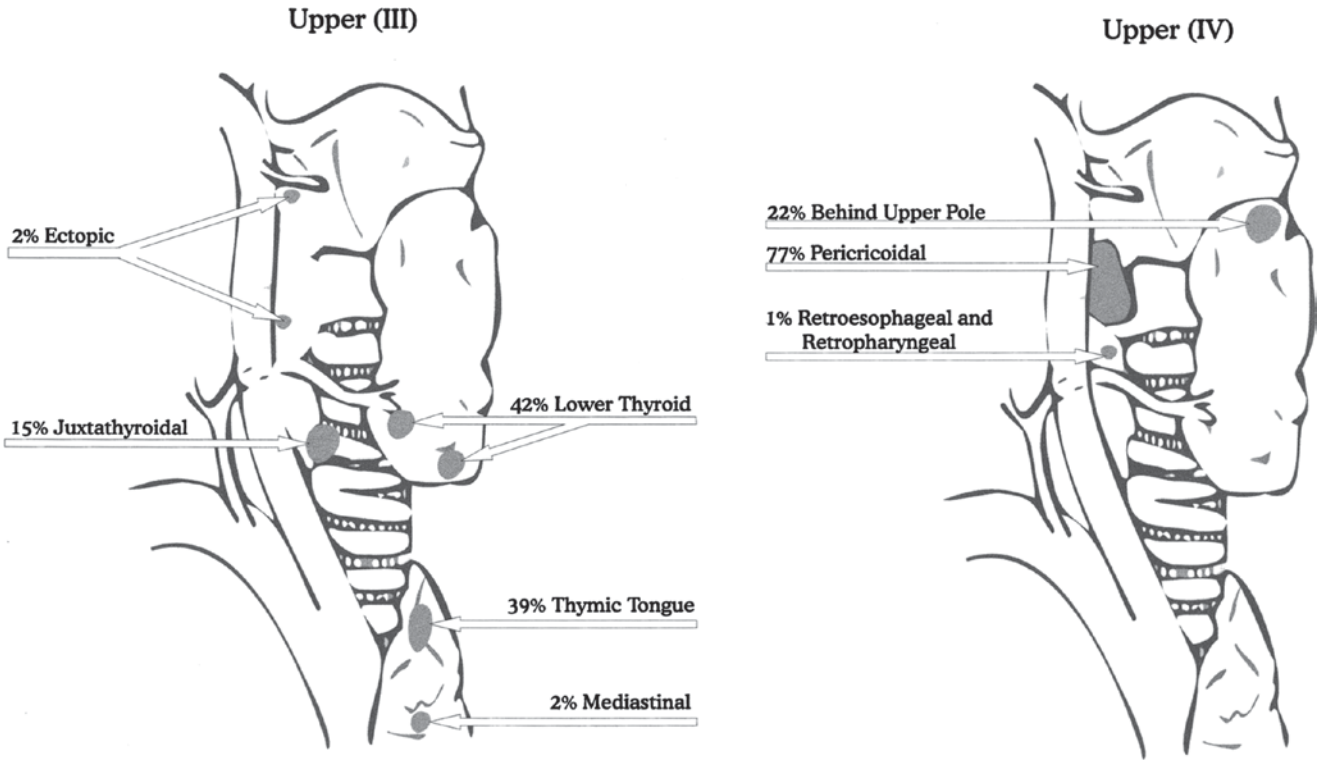

Fig. 7.2 Anatomical distribution of the parathyroid glands with the thyroid lobes reflected anteriorly (adapted from [13])

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 latero-posteriorly or ventro-laterally to the lower pole of the thyroid lobe [12, 13] (Fig. 7.2).

7.2.2 Histology and Ultrastructure

The parathyroid glands are demarcated by a thin connective tissue capsule which 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 about 12 μm in diameter, and has a polygonal clear and finely granular cytoplasm with a 7 μm central round hyperchromatic nucleus with a small nucleolus (Fig. 7.3). Chief cells contain intracytoplasmic PAS-positive glycogen [9] and oil red O positive or sudanophilic droplets of neutral lipids [18, 19] which can also be recognized at ultrastructure together with scattered argyrophil membrane bound secretory granules measuring about 150 nm [20–22] (Fig. 7.4). A decrease in intracytoplasmic lipid is seen in a proportion of 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. 7.5), as well as glandular structures[9, 17, 20]. The lumina from the glandular component may contain eosinophilic PAS-positive colloid-like material which lacks the birefringent oxalate crystals characteristically seen in thyroid colloid under polarization. Amyloid can be found in follicular structures from about 50% normal parathyroids and its frequency increases with age, as well as the presence of parathyroid follicles [23]. Ultrastructurally, glandular lumina contain amorphous granular material and/or non-branching 75–100Å wide amyloid fibers which 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. 7.3). Less eosinophilic transitional cells may occur [9]. At ultrastructure, oxyphil cells

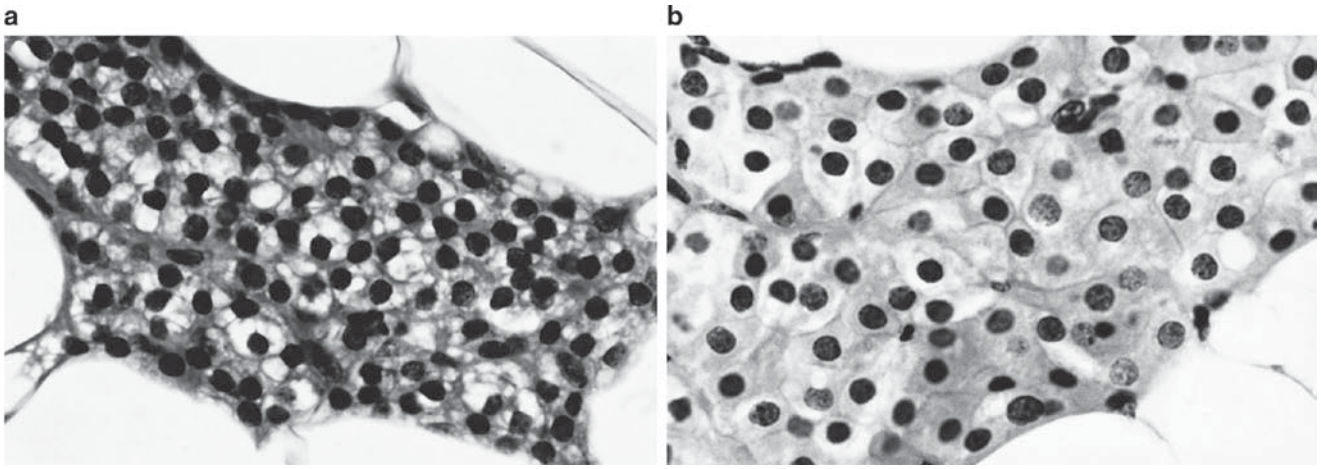

Fig. 7.3 Normal adult parathyroid gland. (**a**) Solid sheet of chief cells with clear vacuolated cytoplasm and regular hyperchromatic nuclei (H&E, ×720). (**b**) Oxyphil cells with well-demarcated granular cytoplasm and regular nuclei (H&E, ×720)

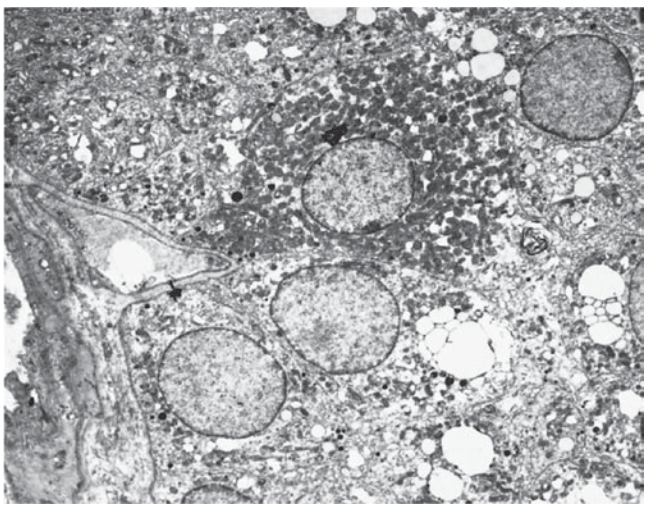

Fig. 7.4 Electron micrograph showing chief cells containing cytoplasmic secretory granules and fat droplets. Note solitary oxyphil cell with abundant intracytoplasmic mitochondria (×9,500)

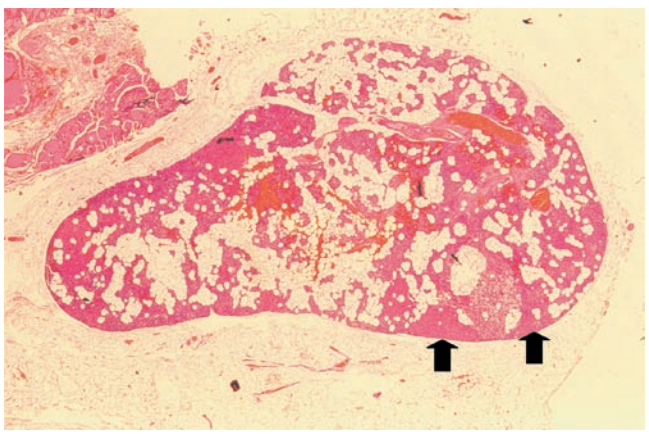

Fig. 7.5 Parathyroid gland from a 66-year-old female showing an almost equal distribution of parenchymal cells and fatty tissue. Note age-related poorly defined nodular conglomerate of clear vacuolated chief cells surrounded by groups of oxyphil cells (*arrows*) (H&E, ×80)

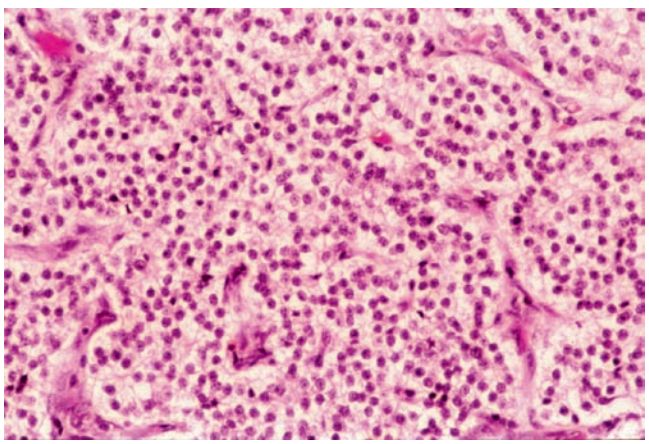

Fig. 7.6 Fetal parathyroid gland. Chief cells with abundant finely vacuolated clear cytoplasm and round eccentric nuclei arranged in solid sheets surrounded by a delicate vascular framework devoid of stromal fat (H&E, ×720)

show abundant mitochondria and fewer organelles and cytoplasmic membrane bound argyrophil granules than chief cells [20–22] (Fig. 7.4). Oxyphil cells increase in number with age (Fig. 7.5), may be found singly or in small clusters from childhood to adulthood, forming nodular conglomerates difficult to distinguish from oxyphil microadenomas in older persons [9, 12], and may also acquire a follicular pattern often containing intraluminal amyloid [9, 23]. It is estimated that oxyphil and transitional cells reach up to 1% of the parenchymal mass in individuals under 40 years and about 5% in elderly people [12, 17]. In the fetus the parathyroid gland is composed of solid sheets of chief cells with abundant, finely vacuolated cytoplasm with round hyperchromatic eccentric nuclei surrounded by delicate vessels and absence of oxyphil and fat cells [8–10] (Fig. 7.6).

Chief cells start to show PTH-immunoreactivity from 10 weeks of gestation [8]. PTH product and PTHmRNA content show an inverse relationship in normal classic 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 cytokeratins and PTHrP [27, 29–31]. No proliferative cell activity is evident by Ki-67 immunocytochemistry [32–34] and the AgNOR technique [35].

The stromal fibrofatty component increases with age, is often irregularly distributed within the parathyroids and frequently 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 about 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 the 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 subjects as compared with healthy ambulatory subjects, in whites as compared with black people [12, 14, 15, 17].

Other rare histological findings in the parathyroids include microcystic structures, scars or fibrous hyalinizing tissue, bone marrow, and salivary gland remnants [9, 12, 17, 36].

7.2.3 Physiology of Calcium Metabolism

PTH is synthesized in the chief cell through different processes before being secreted to the bloodstream by exocytosis. Intact human PTH consists of an 84 amino acids single-chain polypeptide with a molecular weight of approximately 9,600, the 1–34 amino terminal fragment exhibits most of the function. Hypocalcemia is the main secretagogue of PTH mediated through the CaR on the chief cells which also has per se a calcium ion down- or upregulation 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 [1]. 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. The extracellular CaR enables the parathyroid glands and other CaR-expressing cells (e.g., thyroid C-cells, kidney) to sense alterations in the level of calcium and to respond with changes in function that aims to normalize the blood calcium concentration [4].

PTHrP is a homolog non-hormone product of PTH. It activates the PTH1 receptor, is ubiquitously synthesized in normal non-endocrine and endocrine tissues, including the parathyroid, and its secretion is not regulated by serum calcium [1, 38]. 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 [1] and in some pathological conditions when produced in high amounts, such as sarcoidosis [39] and humoral hypercalcemia of malignancy and benignancy [38, 40]. Hyperparathyroidism due to ectopic secretion of PTH by a non-parathyroid tumor is rare [41].

Vitamin D and its metabolites 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D, acting through vitamin D receptors, downregulates the synthesis of PTH in the parathyroid chief cells, increases the absorption of calcium and phosphate in the small intestine and, simultaneously with PTH, activates bone mineral resorption [1, 42]. Hypercalcemia due to dysregulated high vitamin D production by inflammatory cells and granulomas has been described in sarcoidosis [43], *C. 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 non-parathyroid hormone-related hypercalcemia due to sarcoidosis, vitamin D toxicity, lymphoma, and other malignancies [45].

7.3 Developmental and Acquired Abnormalities

7.3.1 Supernumerary Parathyroid Glands

Supernumerary (5 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, though 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].

7.3.2 Ectopic Parathyroid Tissue

Ectopic parathyroid tissue can be situated in places that are within the normal developmental pathway of the parathyroid glands, such as the pharyngeal wall, at or lateral to the carotid sheath, intravagal, 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. 7.7) [9–13, 47, 48]. An excessively enlarged hyperplastic or neoplastic parathyroid may lead to further caudal migration of the gland [49]. Parathyroid IV may also be found fusioned to, or embedded in, thymic tissue IV around or within the thyroid [7] (Fig. 7.7). Ectopic parathyroid tissue within a lymph node of the neck has been reported [50]. Any lesion that primarily affects the parathyroids may occur in ectopic parathyroid tissue and produce hyperparathyroidism or, more rarely, compressive symptoms like vocal cord palsy in the case of intravagal parathyroid, for example [46, 49, 51–55]. The commonest situation during surgery is that in which three normal or hyperplastic parathyroid glands are found and 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 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]. Ectopic parathyroid tissue is easier to find in patients with parathyroid hyperplasia (up to 36% of the cases) [46, 56] than in cadavers (up to 11%) [11–13] as the glands are obviously larger.

Ectopic parathyroid tumors or hyperplasia, 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. 7.8). These include follicular adenoma and carcinoma (classic, oncocytic, or clear cell types), hyalinizing trabecular tumors, and C-cell neoplasms. Immunoreactivity for thyroglobulin, calcitonin and TTF1, and negative PTH expression will favor their thyroid origin [57]. Polarization may prove helpful to identify birefringent oxalate crystals that may be seen in colloid from thyroid follicles or, inversely, amyloid fibers that may occur in lumen from 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. Other neck tumor that may pose problems in differential diagnosis with parathyroid lesions is the paraganglioma which shows nests of chromogranin A immunoreactive/PTH negative chief cells surrounded by S-100 protein positive substentacular cells. Metastatic cervical lymph nodes can be replaced by a variety of malignancies that may resemble a parathyroid lesion, including thyroid and renal cell carcinoma, which should also be differentiated from the rare ectopic normal parathyroid tissue within cervical lymph node [50]. 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. The latter should always be carefully analyzed to avoid overlooking a parathyroid lipoadenoma (*see* below). PTH immunocytochemistry will help in differential diagnoses.

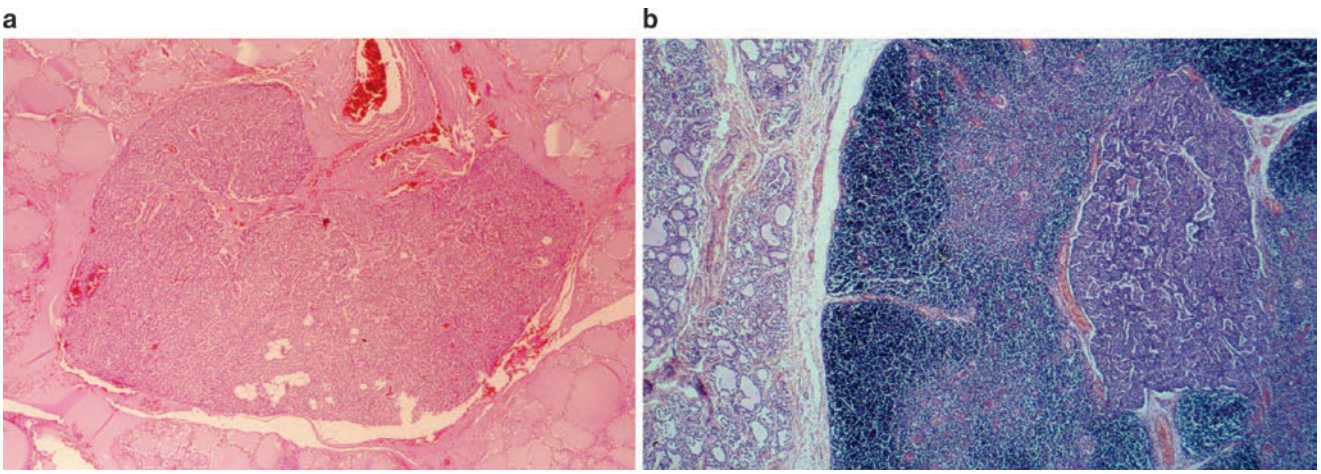

Fig. 7.7 (**a**) Intrathyroid parathyroid gland in a female aged 23 years (H&E, ×20). (**b**) Intrathyroid parathyroid gland surrounded by thymic tissue in a fetus (H&E, ×60)

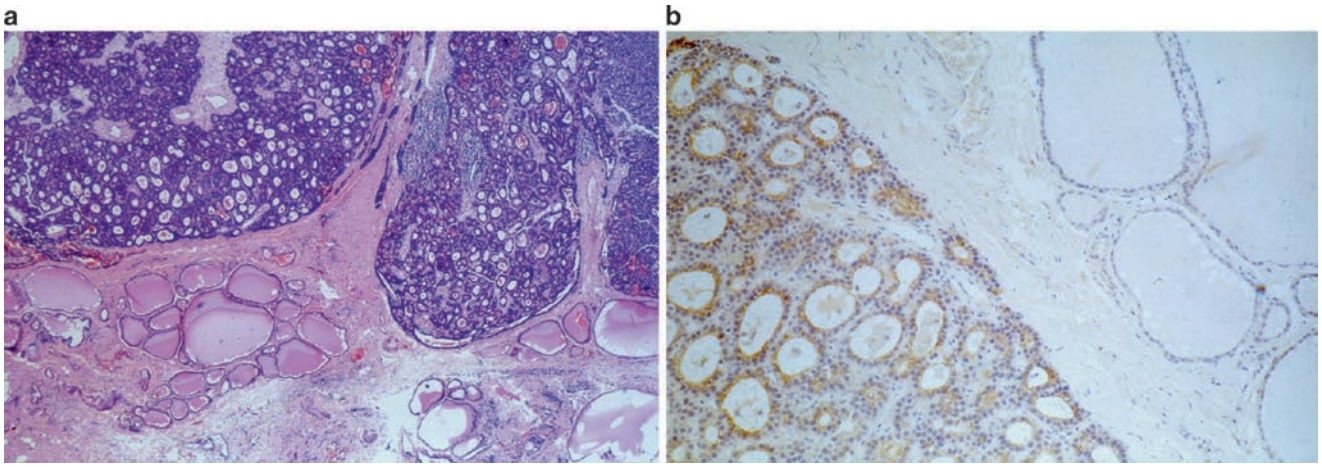

Fig. 7.8 Intrathyroid parathyroid chief cell adenoma. (**a**) The tumor is entirely composed of follicular structures. Note tiny nests of normal looking parathyroid parenchymal tissue at periphery of the tumor (H&E, ×20). (**b**) PTH immunoreactivity in tumor cells adjacent to non-reactive thyroid follicles (immunoperoxidase, ×180)

7.3.3 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. Another situation is 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 [61, 62]. In post-parathyroidectomy implants, seeded parathyroid cell nests can be found lying within fibrofatty tissue which may be adjacent to suture granulomas [62] or birefringent foreign body 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 and PTH and cytokeratin negative paraganglionic chief cells surrounded by S-100 protein immunoreactive sustentacular cells [63].

7.3.4 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 which usually correlates with functional phosphocalcic status of the patient, ranging from normal looking 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 PTHmRNA [64]. Hyperplastic areas can be seen admixed with striated muscle fibers and fibrofatty tissue resembling invasion (Fig. 7.9). Development of a parathyroid carcinoma in a parathyroid implant may occur and absolute criteria of such a diagnosis include vessels or perineural 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].

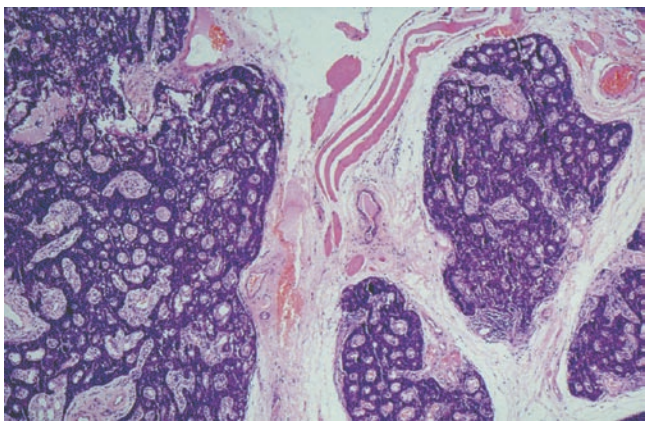

Fig. 7.9 Parathyroid graft. Chief cell nodular hyperplasia admixed with skeletal muscle fibers in a patient with recurrent tertiary hyperparathyroidism (H&E, ×80)

7.3.5 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 are predominant in females with a wide age range at presentation, are usually asymptomatic, and often present as palpable solitary cold "thyroid" nodule. Intracystic hemorrhage may lead to painful neck swelling [68–70]. Intrathyroid parathyroid cyst is rare [71]. The diagnosis of a parathyroid cyst can be achieved by ultrasound-guided fine needle aspiration. The aspirated fluid is usually water-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 which is usually lined by single or stratified cuboidal to flattened epithelium [68, 69, 73] (Fig. 7.10).

Differential diagnosis between parathyroid cyst and cystic parathyroid adenoma may be difficult on pure morphological grounds. Most parathyroid cysts are considered 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 wall 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 like branchial cleft cysts but are 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, 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 often the result of infarction or hemorrhage of a thyroid nodule or adenoma, and the 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 completely a lymph node of the neck and present just as uni- or multi-loculated 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 multi-loculated, 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. 7.11). Cartilage, thymus IV and salivary gland remnants have been described in association with these cysts [75].

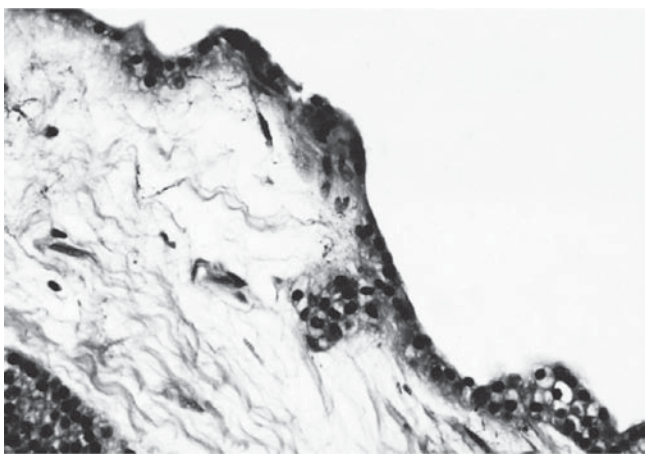

Fig. 7.10 Parathyroid cyst. Cyst wall lined by chief cells (H&E, ×360)

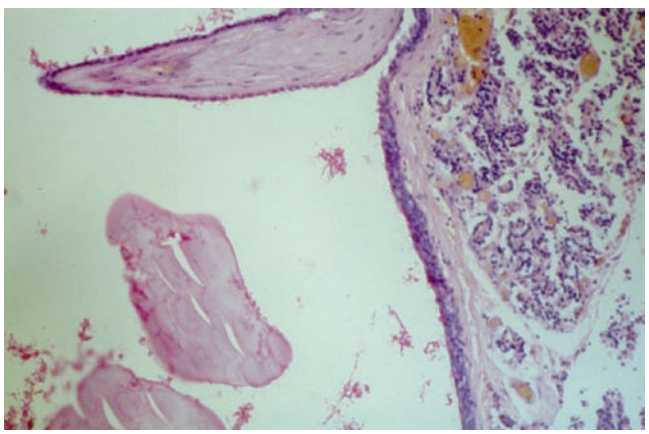

Fig. 7.11 Ultimobranchial cyst from an individual with undescended lingual thyroid. Parathyroid IV adjacent to cyst wall with incomplete fibrous septae and lined by respiratory type epithelium (H&E, ×80)

7.3.6 Parathyroid Aplasia or Hypoplasia (DiGeorge's Syndrome)

DiGeorge's (or velocardiofacial) syndrome is an autosomal dominant congenital (22q11.2 deletion) developmental anomaly characterized by total or partial absence of the thymus and parathyroid glands along with cardiovascular and craniofacial malformations (*see* Hypoparathyroidism).

7.4 Primary Hyperparathyroidism

Primary hyperparathyroidism refers to an increase of serum calcium and PTH concentrations, and is one of the most common endocrine diseases. Its clinical presentation has dramatically changed in the last 40 years, and now the disease typically affects elderly women and is characterized by mild hypercalcemia and few traditional classic manifestations such as bone pain due to osteitis fibrosa cystica (von Recklinghausen's disease) and symptoms of renal lithiasis. The change in clinical presentation was largely caused by the development of automated serum calcium measurement in the late 1960s that made possible the identification of a large number of "asymptomatic" patients (about 50% of the cases) with additional vague symptoms, such as muscular weakness, malaise, arterial hypertension, mental depression, and mild osteoporosis [1, 76, 77]. In 1975, the incidence of primary hyperparathyroidism in Rochester was in excess of 100 cases per 100,000 patient years as compared with 15.8 cases in earlier decades. More recently, the incidence of primary hyperparathyroidism has fallen to approximately 15 cases per 100,000 patient years for reasons that are not clear so far [77]. From a recent study in Europe, a 21/1,000 primary hyperparathyroidism prevalence was found in women aged 55–75 years, which is equivalent to 3/1,000 prevalence in the general population, and with a predominance over men in a proportion of about 3:1. This led also to a five-fold increase in the apparent incidence of primary hyperparathyroidism for the identification of patients who were never diagnosed before (catch-up effect) [76].

Primary hyperparathyroidism may result from parathyroid adenoma (single gland disease) (80–85%), hyperplasia (multi-glandular disease) (10–15%) or carcinoma (less than 1%) [77]. Parathyroid adenomas incidentally found during a neck surgical procedure unrelated to parathyroidectomy show a prevalence of 1.2% [78]. Diffuse or nodular parathyroid gland enlargement is found in about 1% of thyroidectomized female patients [79]. These probably represent early subclinical forms of hyperparathyroidism where the parathyroid glands are usually smaller than those found in patients with preoperatively proved primary hyperparathyroidism [78, 79]. Around 10% of patients on lithium therapy for psychiatric disorders become hyperparathyroid due to parathyroid adenoma or, less frequently, hyperplasia [80, 81]. Primary hyperparathyroidism occurring at young age should raise the suspicion of antecedents of neck irradiation in childhood with consequent parathyroid tumor generation [82] or a familial syndrome (Table 7.1).

Hyperparathyroidism due to an ectopic secretion of PTH by a non-parathyroid tumor is rare [41]. It is more likely that a non-parathyroid tumor secreting PTHrP will exert a PTH-like endocrine effect, leading to humoral hypercalcemia of malignancy or benignancy [38, 40].

Table 7.1 Genetics and pathology of autosomal dominant familial hyperparathyroidism syndromes.

Syndrome	Gene (Locus)	Parathyroid histology	Other findings
FHPT-JT	HRPT2 (1q25-32)	Adenoma, carcinoma	Ossifying fibroma of the jaw, renal cysts, hamartoma, carcinoma and Wilms tumor.
FIHPT	MEN 1 (11q13)	Hyperplasia	
	HRPT2 (1q21-23)	Adenoma, carcinoma	
	CaR (3q.13-q21)	Hyperplasia	
MEN 1	MEN 1 (11q13)	Hyperplasia	Pancreatic, pituitary and gut endocrine hyperplasia/tumors, adrenal cortical tumors, lung and thymic carcinoids, multiple lipomas, facial angiomas and collagenomas.
MEN 2A	RET (10q11.2)	Hyperplasia	C-cell hyperplasia, thyroid medullary carcinoma, pheochromocytoma.
FBHH	CaR[a] (3q.13-q21)	Normal Mild hyperplasia Lipohyperplasia	
NSHPT	CaR[b] (3q.13-q21)	Mild hyperplasia	

[a]heterozygous

[b]homozygous inactivating mutations

MEN multiple endocrine neoplasia; *FHPT-JT* familial hyperparathyroidism-jaw tumor syndrome; *FIHPT* familial isolated hyperparathyroidism; *FBHH* familial benign hypocalciuric hypercalcemia; *NSHPT* neonatal severe hyperparathyroidism; *CaR* calcium sensing receptor gene

7.4.1 Familial Hyperparathyroidism

Less than 5% of cases presenting with hyperparathyroidism are familial and encompass a clinically and genetically heterogeneous manifestation of autosomal dominant traits, such as hyperparathyroidism-jaw tumor syndrome (HPT-JT), multiple endocrine neoplasia type 1 (MEN1), multiple endocrine neoplasia type 2A (MEN2A), familial hypocalciuric hypercalcemia (FHH), and familial isolated hyperparathyroidism (FIHPT) (Table 7.1).

HPT-JT syndrome is characterized by hyperparathyroidism, jaw ossifying fibromas (30%), and renal neoplastic and non-neoplastic abnormalities. This trait is linked to germline mutations of the HRPT2 oncosuppressor gene on chromosome 1q25-32 that encodes the protein parafibromin. The pathology of parathyroids includes multiple adenomas, some are cystic, and 10–15% of the patients develop parathyroid carcinoma [77, 83].

MEN1 is associated with proliferative lesions of the parathyroid glands (90%), gastroenteropancreatic system (60%), and pituitary gland (30%). This trait is linked to germline mutations of the MEN1 tumor suppressor gene on chromosome 11q13 that encodes the protein menin. Other associations include lung and thymic neuroendocrine tumors, adrenal cortical tumors, multiple lipomas, and facial angiomas and collagenomas [77]. MEN1 is found in up to 17% patients with primary hyperparathyroidism and in up to 43% with primary parathyroid chief cell hyperplasia. Hyperparathyroidism is the commonest manifestation of MEN1 and by age 40 its prevalence approaches 100%. The age at diagnosis in screening programs is about 20 years [84–87].

MEN2A is associated with thyroid C-cell hyperplasia, medullary carcinoma, pheochromocytoma, and parathyroid hyperplasia linked to germline *RET* point mutations on 10q11.2. The prevalence of parathyroid disease in this setting is about 20–30%, 0.3% in patients with primary hyperparathyroidism and 1% in cases with primary parathyroid chief cell hyperplasia. The mean age at diagnosis of 38 years may present with single or multiple parathyroid gland enlargements at initial operation and is found concomitantly with thyroid medullary carcinoma or pheochromocytoma in 77% of the cases [86, 88–90].

FHH results from a heterozygous germline inactivating mutation of the CaR gene on 3q13.3-q21 and is characterized by mild hypercalcemia, high PTH levels, and low urinary calcium excretion. Neonatal severe primary hyperparathyroidism, a homozygous type of the disease, shows a clinically more aggressive phenotype [4, 84]. Rare forms of FHH are linked to mutations on chromosome 19 [4]. The parathyroid glands in FHH present normal histology, mild hyperplasia, or lipohyperplasia [91–93] (Table 7.1).

Familial isolated hyperparathyroidism (FIH) is a subgroup that can result from an incomplete expression of a heritable trait like MEN1, FHH, and HPT-JT syndrome [77, 83].

7.4.2 Parathyroid Adenoma

The commonest cause of primary hyperparathyroidism (80–85%) 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 in patients on lithium therapy for psychiatric disorders [80, 81], after low dose external neck irradiation and have also been reported after radioiodine therapy for Graves' disease [97, 98]. 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 years [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 (5%), MEN1 (25–40%), p53, and GNAS1 genes, respectively, loss of heterozygosity on chromosomes 1p, 6q, 9p, 11q (most frequent),11p, 13q, and 15q and gains in 7, 16p, and 19p [77, 84, 99].

Parathyroid adenomas are more common in the lower parathyroid glands and vary considerably in size (few millimeters – 10 cm) and weight (0.025–120 g) [51, 73, 77, 95, 100]. The average size of adenomas from patients without significant bone disease is around 0.5–1 g. 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 especially in large adenomas. A rim of normal parathyroid tissue is sometimes evident around the tumor [51, 73, 77] (Fig. 7.12). Microscopically, parathyroid chief cell adenomas may show a wide cytoarchitectural

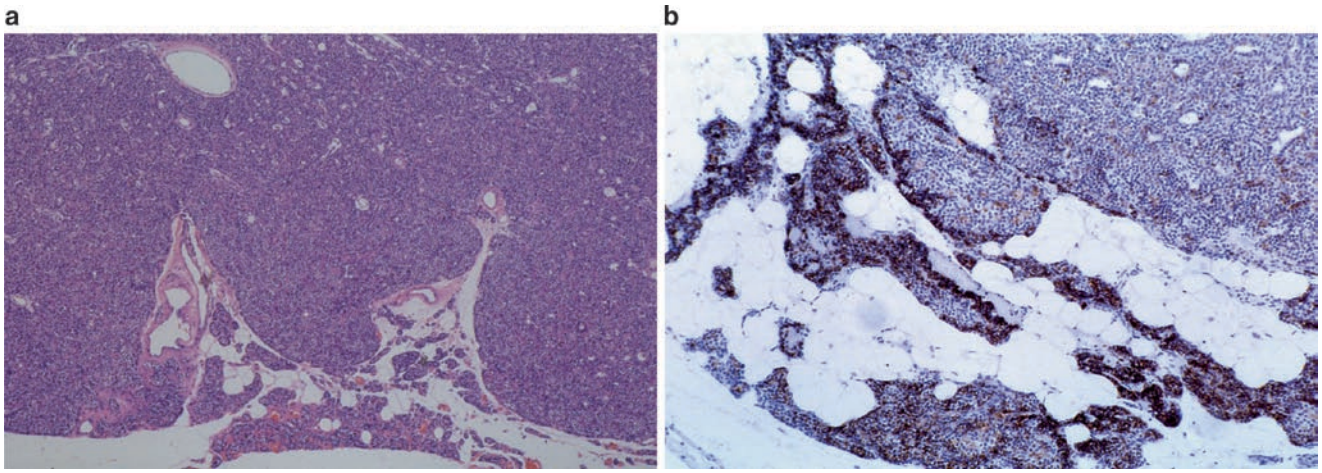

Fig. 7.12 Parathyroid adenoma. (**a**) Unencapsulated classic adenoma of chief cells arranged in a solid pattern and surrounded by a rim of normal parathyroid tissue (H&E, ×20). (**b**) PTH weak immunoreactivity in tumor cells next to strongly positive suppressed normal parathyroid tissue (Immunoperoxidase, ×180)

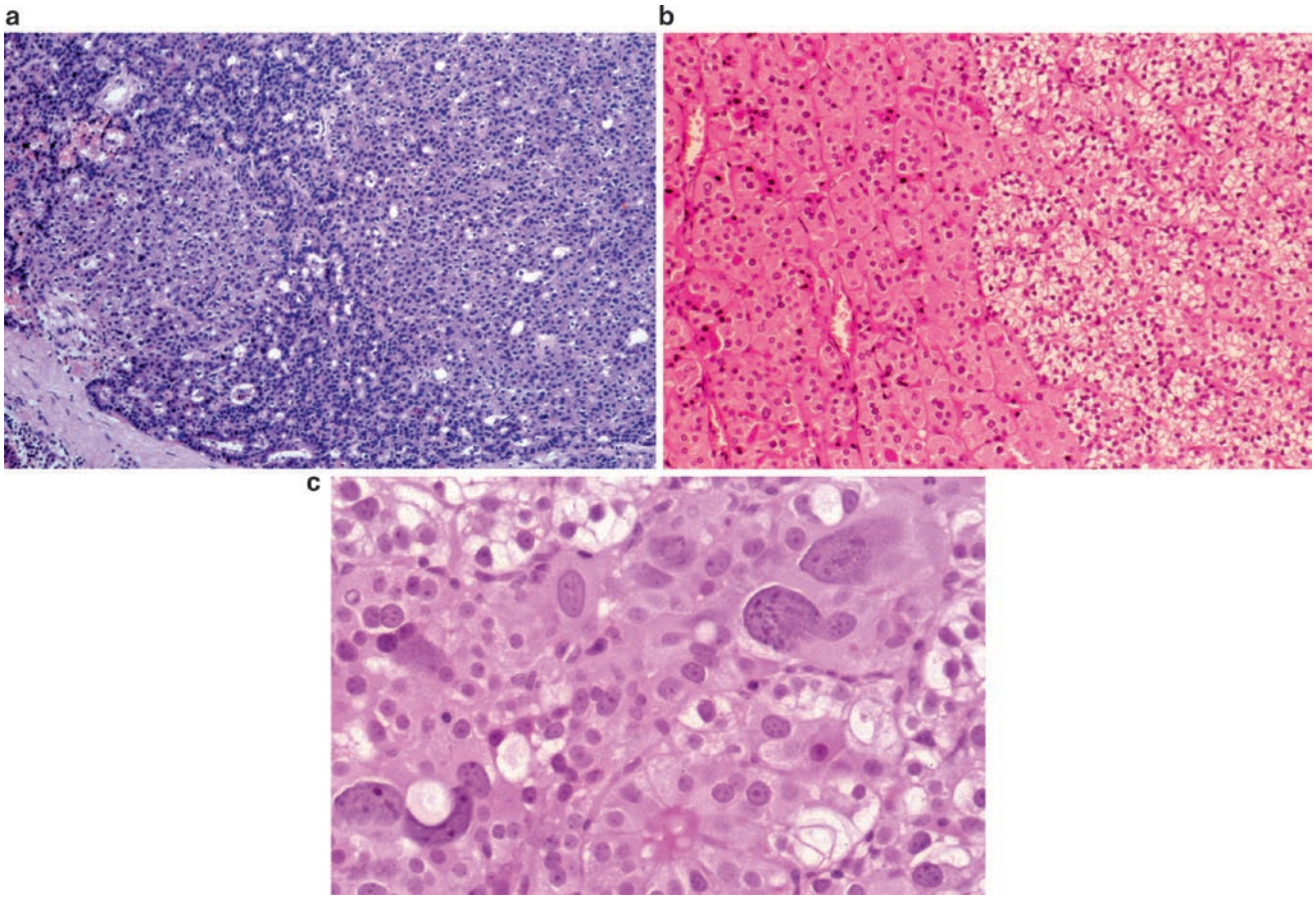

Fig. 7.13 Parathyroid adenoma. (**a**) Trabecles and follicles of chief cells and transitional cells (H&E, ×120). (**b**) Solid arrangement of chief cells and oncocytic cells (H&E,×180). (**c**) Nuclear pleomorphism (H&E, ×360)

spectrum [101] (Figs. 7.13 and 7.14). The nuclei are often regular rounded hyperchromatic and usually larger than those from the adjacent normal parathyroid tissue. Large pleomorphic nuclei occur in about 10% of the cases (Fig. 7.13), as well as multi-nucleate chief cells [77, 102]. Oxyphil cells may occur as a minor component admixed with chief cells (Fig. 7.13). The neoplastic cells can be arranged in solid, trabecular, follicular, microcystic, papillary, and peripheral

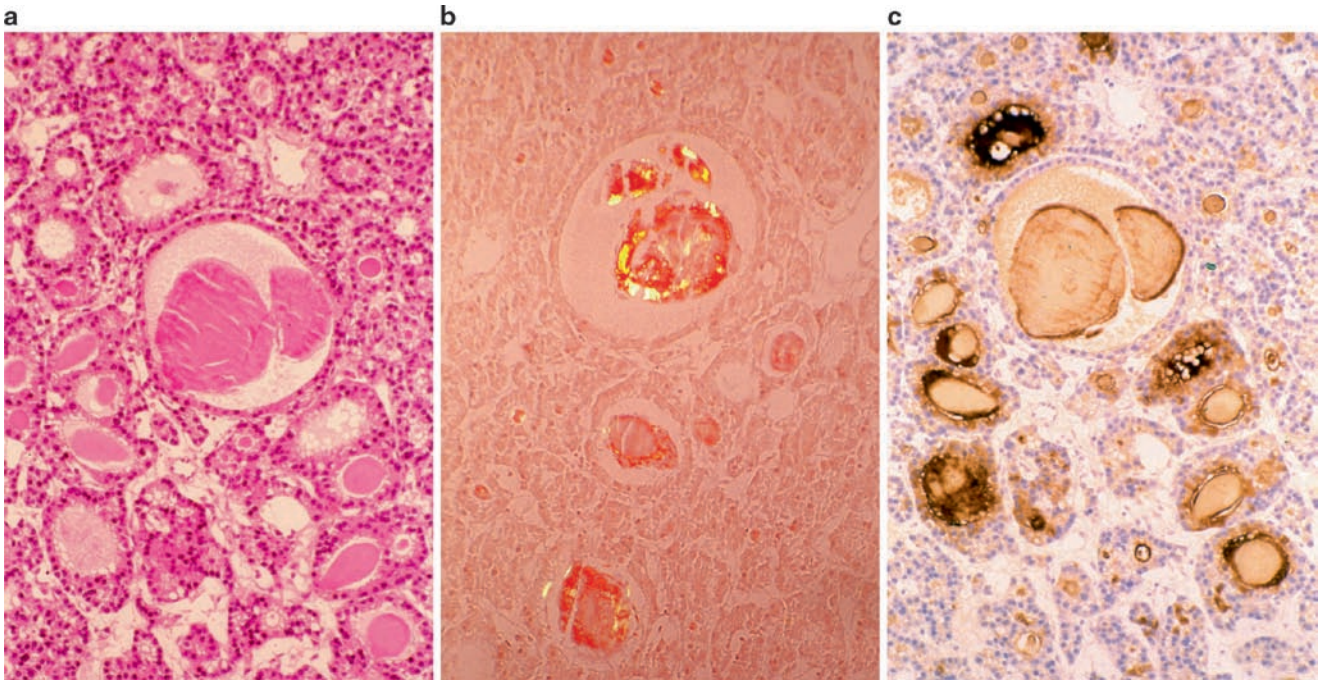

Fig. 7.14 Parathyroid adenoma. (**a**) Follicles lined by chief cells with colloid-like material and dense eosinophilic amyloid that displays (**b**) apple green birefringence after polarization and (**c**) PTH-immunoreactivity which is also present in some neoplastic cells (H&E, Congo red and immunoperoxidase, respectively, ×450)

palisading structures, or an admixture [12, 51, 73, 103] (Figs. 7.8, 7.12, 7.13 and 7.14). Mitotic figures may be present in up to 70% of the cases, and little (less than 4%) or no proliferative cell activity is evident by Ki-67 immunocytochemistry [32–34, 77] and the AgNOR technique [35]. Adenomas may show a high mitotic rate, particularly in cases with hyperparathyroid crisis [104]. Lumina from follicular structures may be empty, show colloid-like material or contain amyloid [23, 25] (Fig. 7.14). Cystic changes in an adenoma may be genuine or occur after tumor infarction (Fig. 7.15). 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]. Severe fibrosis may occur after fine needle biopsy or parathyroidectomy by percutaneous ethanol injection [105, 106]. Parathyroid adenomas replaced by destructive lymphocytic infiltrates resembling chronic parathyroiditis have been described [107]. A rim of "normal" parathyroid tissue can be found around the tumor capsule or merging with neoplastic cells (Fig. 7.12), or may not be identifiable when the gland is totally replaced by tumor [73]. Often chief cells from the "normal" suppressed parathyroid tissue are slightly smaller with crowded nuclei, contain abundant PAS-positive glycogen granules that are also identifiable at ultrastructure [20], and show intense PTH immunoreactivity with low PTHmRNA 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]. Cells from parathyroid adenomas show a varying extent of argyrophilia, cytokeratin, chromogranin and PTH immunoreactivity, and PTHmRNA expression [21, 26, 27, 30, 77]. Ultrastructurally, the adenomatous cells show features of chief cell differentiation, including membrane bound secretory granules [20, 22].

The main differential diagnoses of parathyroid adenoma are parathyroid carcinoma – which shows unequivocal 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 [77]. In this case, the possibility of antecedents of neck irradiation or a genetically determined defect [82, 83, 88, 108] must be considered. Cyclin D1 overexpression (20–40%), DNA ploidy, AgNOR enumeration, galectin-3, and proliferative cell rate as shown by Ki-67 may show overlapping results making these markers and methods of little value in their differential diagnosis [32, 35, 77, 84]. Parafibromin and RB protein immunoreactivity usually shows strong diffuse nuclear expression in parathyroid adenomas and is generally absent or reduced in parathyroid carcinoma [77, 84, 108]. Parafibromin is usually absent or reduced in parathyroid adenoma linked to HPT-JT [77, 108]. Care should be taken to avoid a mis-diagnosis of parathyroid carcinoma in parathyroid adenomas with severe intratumoral and periparathyroideal

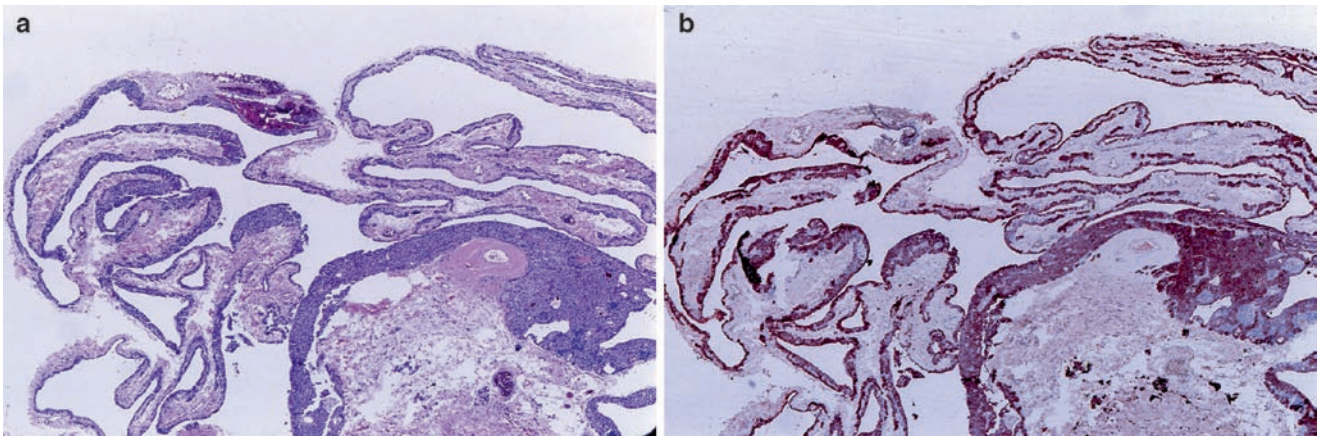

Fig. 7.15 Cystic adenoma. (**a**) Cyst wall lined by neoplastic chief cells which also show focal solid architectural pattern of growth (H&E, ×40). (**b**) PTH immunoreactivity in chief cells (Immunoperoxidase, ×40)

fibrosis after fine needle biopsy or percutaneous ethanol injection [104, 105].

7.4.3 Variants of Parathyroid Adenoma

7.4.3.1 Cystic Adenoma

This variant may be difficult to differentiate morphologically from a parathyroid cyst and should not be confused with ordinary parathyroid adenomas that may contain a minor cystic component. Adenomas can be originally cystic and are relatively common in familial HPT-JT [77, 83]. Most cystic adenomas originate as a result of infarction and consequent hemorrhage in a pre-existing adenoma [68]. 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 which 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* before) [68–70].

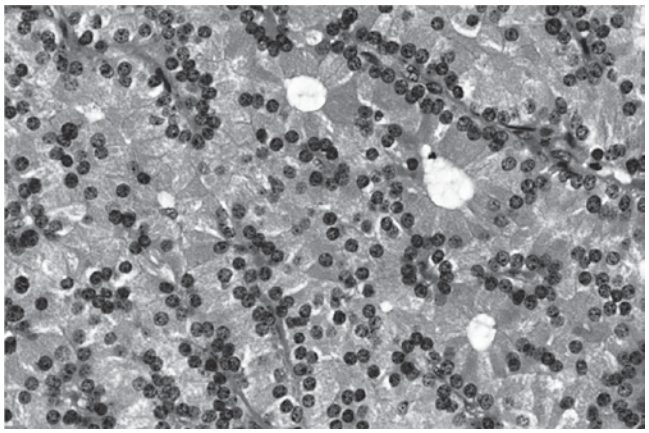

Fig. 7.16 Oncocytic adenoma. Trabecles and follicles of well-demarcated cells with abundant granular eosinophilic cytoplasm and regular hyperchromatic nuclei (H&E, ×450)

7.4.3.2 Oncocytic Adenoma

Less than 8% of hyperfunctioning parathyroid adenomas are oncocytic. They may occasionally present with symptoms of severe hyperparathyroidism resembling parathyroid carcinoma [109]. The tumors are macroscopically tan in color and well circumscribed. Histologically, they are composed of at least 90% of cells with abundant granular eosinophilic cytoplasm with regular central nuclei and prominent nucleolus arranged in trabecular, solid, or glandular patterns (Fig. 7.16). Nuclear pleomorphism and multi-nucleate cells may occur. The tumor cells usually show focal PTH immunoreactivity. At ultrastructure, cytoplasmic mitochondria are numerous and are admixed with few scattered secretory granules [51, 73, 77, 109–111].

7.4.3.3 Lipoadenoma (Hamartoma)

Parathyroid lipoadenoma may occur with hyperparathyroidism and present as a neck mass. Lipoadenomas vary in weight and measure up to several centimeters [112–114]. The first reported tumor case 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 greater than 90%. Lipoadenomas are composed of chief cells, with or without oxyphil cells, arranged in solid, trabecular, and glandular patterns, or an admixture, surrounded by prominent fatty tissue component resembling the normal adult parathyroid gland [112–114] (Fig. 7.17). Pure oxyphil lipoadenoma may occur [113] (Fig. 7.17). The stroma of parathyroid lipoadenomas may show inflammatory cells, calcium deposits, and myxomatous changes which occasionally could be prominent with little interspersed lipomatous component (myxoid lipoadenoma) [115]. The main differential diagnosis of parathyroid lipoadenoma is with lipoma [114] and its distinction from lipohyperplasia (*see* below) will depend on the number of glands affected.

7.4.3.4 Mixoadenoma

This neoplastic lesion probably represents a subset of lipoadenoma with myxoid stroma, unaccompanied by fatty tissue component [116]. Lipoadenoma with large myxoid stroma and little fatty tissue component has been regarded as myxoid lipoadenoma [115].

7.4.3.5 Water-Clear Cell Adenoma

This rare tumor shows nests and glandular structures of PTH-immunoreactive microvacuolated cells with abundant well demarcated clear cytoplasm (Fig. 7.18). 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 which 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, TTF1, and calcitonin immunocytochemistry would play an important role in their differential diagnosis, and between primary thyroid clear-cell neoplasms and intrathyroid water-clear cell parathyroid adenomas [120].

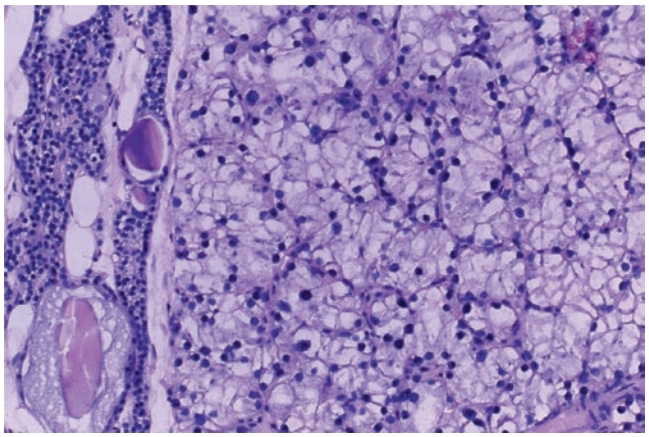

Fig. 7.18 Water-clear cell adenoma. Nests of neoplastic water-clear cells surrounded by a rim of normal parathyroid tissue (H&E, ×80)

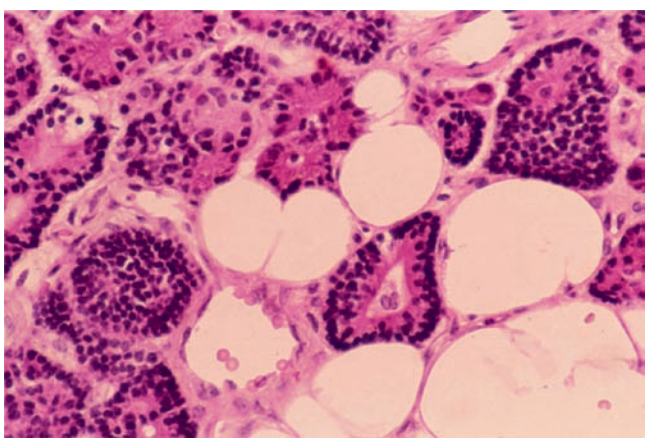

Fig. 7.17 Lipoadenoma. Nests and follicles of chief cells and oncocytic cells admixed with adipose tissue (H&E, ×80)

7.4.3.6 Microadenoma

Parathyroid microadenomas show an incidence of 1% in all operations for primary hyperparathyroidism. These are defined as single lesions smaller than 6 mm and weighing less than 0.1 g that occur in externally undeformed parathyroid glands [77, 95]. The high incidence is probably due to the 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. Multiple sections may be required to identify a parathyroid microadenoma in an otherwise normal parathyroid gland [29]. Microadenomas are composed of nests of chief cells or oxyphil cells that may present as a small non-encapsulated 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 [77, 95, 100]. The oil red O method during frozen section, high PTHmRNA expression in the tumor on paraffin embedded tissue and correlating the findings with the effect of surgery on serum calcium levels might help to interpret tissue functionality.

7.4.3.7 Atypical Adenoma

These tumors show equivocal, borderline, or suspicious features of parathyroid carcinoma. These include intratumoral banding fibrosis, mitotic activity, trabecular growth, and presence of tumor cells in the surrounding capsule, but lack unequivocal evidence of malignancy, such as invasion to peritumoral vessels, perineural spaces, and surrounding soft tissues and neck structures. Interestingly, these tumors present with calcium levels and a molecular phenotype intermediate between those of adenomas and carcinomas [77].

7.4.4 Parathyroid Carcinoma

Parathyroid carcinoma accounts for less than 1% of cases of primary hyperparathyroidism. Most parathyroid carcinomas are sporadic, but they have also been reported to occur in association with parathyroid hyperplasia from primary, secondary and tertiary hyperparathyroidism, familial isolated hyperparathyroidism, familial HPT-JT (10–15%) (Table 7.1) and after neck irradiation. The majority of sporadic parathyroid carcinomas are associated with somatic mutation of the RB and HRPT2 genes and cyclin D1 overexpression, while a few of them may show p53 mutation [77, 84, 121, 122]. Comparative genomic hybridization and fluorescense in situ hybridization studies have demonstrated several chromosome losses and gains in parathyroid carcinoma [77, 84], a topic that is not within the scope of this chapter.

The patients usually present with symptoms of severe hyperparathyroidism and show a wide age range with a peak in the fifth and sixth decades and almost equal sex distribution. A high proportion of cases (30–76%) have a palpable mass in the neck, sometimes with laryngeal nerve palsy, and suffer from bone (44–91%) and or renal disease (32–60%) at diagnosis [121]. Parathyroid cancer may rarely present as painful goiter (pseudothyroiditis) [123]. Less than 5% of the tumors are asymptomatic with hypercalcemia discovered on routine serum calcium screening [121]. 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 years survival rate varies from 40 to 86%, with a recently reported 10-year survival rate of 49% in a large series [121, 124].

The majority of parathyroid carcinomas weigh between 2 and 10 g, and measure from 1.3 cm to several centimeters in greatest diameter with a median size of 3.3 cm [54, 102, 124]. The tumors are ovoid or lobulated in shape, sometimes showing a ragged external surface with adhered fat, striated muscle, fibrous, nerve or thyroid tissue (Fig. 7.19). 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 component 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. 7.20). Nuclei may contain cytoplasmic inclusions and show marked pleomorphism. An admixture of chief cells and oxyphil cells may occur [102, 125, 126]. The mitotic rate is usually high but may be absent. Necrosis may occur. At ultrastructure, neoplastic cells show prominent nucleoli and cytoplasmic features of parathyroid chief cell differentiation, often with a small number of secretory granules [20]. Oncocytic parathyroid carcinomas (80% or more oxyphil cell component) do not differ clinically and architecturally from chief cell parathyroid carcinoma, except that the cells show abundant granular eosinophilic cytoplasm (Fig. 7.20)

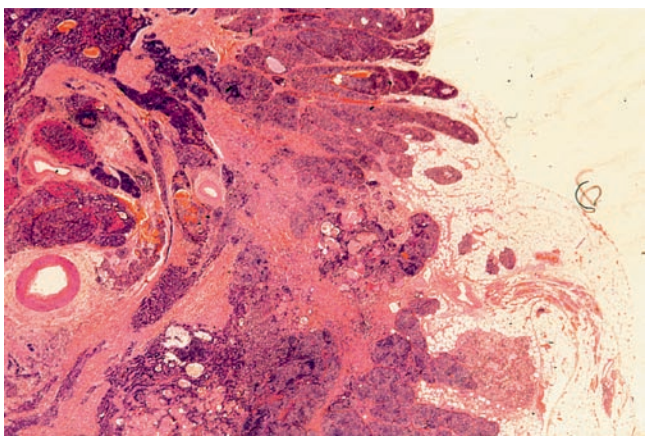

Fig. 7.19 Parathyroid carcinoma. Tumor with ragged edges showing fibrous septae and irradiating growth pattern of invasive islands of neoplastic chief cells deeply infiltrating surrounding soft tissue structures (H&E, ×20)

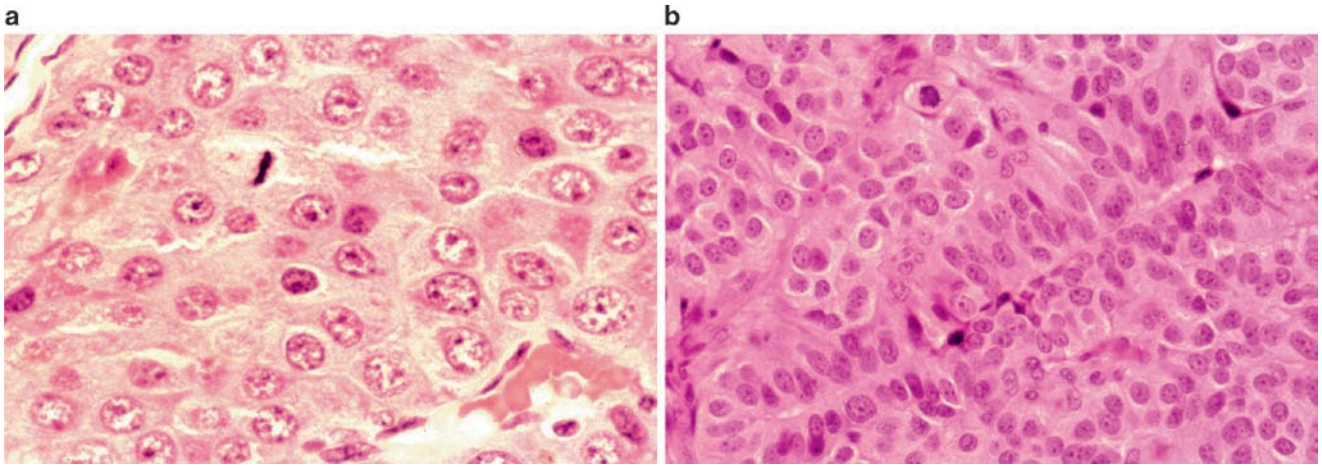

Fig. 7.20 Parathyroid carcinoma. a) Solid growth pattern of chief cells with large, slightly irregular nuclei and prominent nucleoli (H&E, ×720). b) Oncocytic polygonal and elongated neoplastic cells arranged in a trabecular/solid pattern (H&E, ×720). Note mitosis in both fields

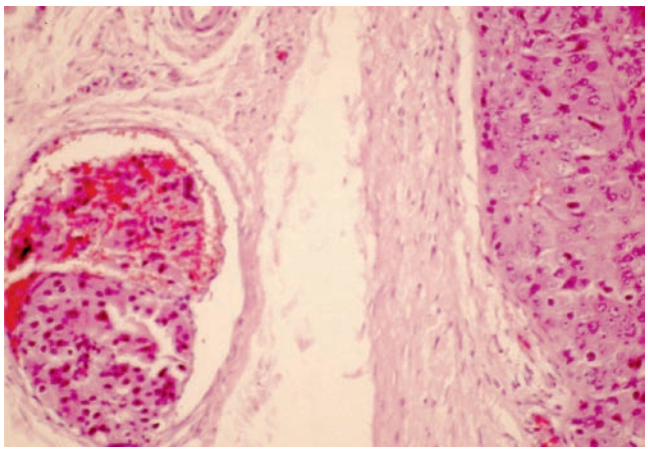

Fig. 7.21 Blood vessel invasion in parathyroid carcinoma (H&E, ×600)

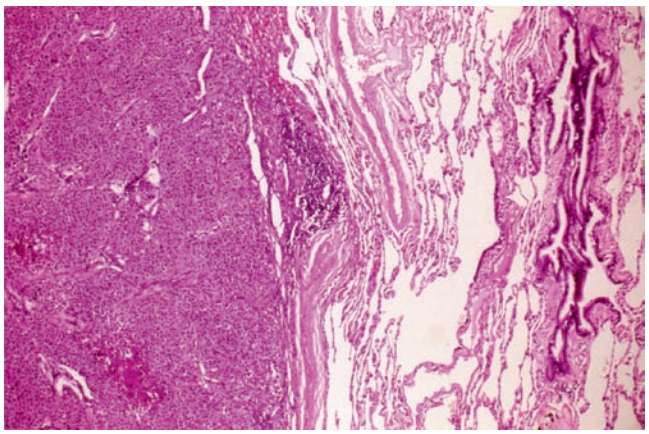

Fig. 7.22 Lung metastasis of parathyroid carcinoma (H&E, ×450)

due to abundant mitochondria content [127, 128]. Parathyroid carcinomas usually show dense fibrous septae occupied by tumor cell islands which can also be found in the tumor capsule invading blood or lymphatic vessels, nerve fibers, and adjacent neck structures [54, 102, 125] (Figs. 7.19 and 7.21). Orcein stain for elastic fibers or endothelial cell immunocytochemical markers may prove useful for demonstrating vascular invasion. PTH and/or PTHmRNA expression could be essential for identifying classic, non-functioning, metastatic, and unusual types of parathyroid carcinomas [26, 129, 130] (Fig. 7.22). Parathyroid carcinoma usually shows negative or reduced focal or diffuse RB protein and parafibromin immunoreactivity [77, 84, 108, 121]. p53 expression was found in a few parathyroid carcinomas that showed no RB mutation, but was also occasionally found in adenomas [84, 121]. Parathyroid carcinoma with sarcomatoid differentiation (carcinosarcoma) has been described [131].

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 [32, 35, 77, 121, 125]. Preliminary studies have shown that telomerase activity seems to be restricted to carcinomas [132]. Absolute criteria of malignancy include lymphatic or vascular invasion, invasion to adjacent soft tissue and 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 and absent or reduced RB protein and parafibromin immunoreactivity [77, 108, 121]. In the absence of unequivocal invasion, a diagnosis of atypical adenoma [77] should be made and a close follow-up advocated.

A surgical report of fixation of the tumor to surrounding neck structures would support a diagnosis of low-grade malignancy during frozen section. Pathologists must be aware that necrosis, bands of fibrosis, adherence to surrounding neck structures, and even laryngeal nerve palsy may occur in parathyroid adenomas following fine needle aspiration and therapeutic percutaneous ethanol injection [104, 105]. Normal parathyroid tissue inclusions in cervical lymph nodes should not be mistaken with metastatic parathyroid carcinoma [50]. Other differential diagnoses where immunocytochemistry would hold good include paraganglioma (negative for cytokeratin and PTH), metastatic renal cell carcinoma (negative for PTH), and thyroid C-cell and follicular cell (oncocytic and clear cell) carcinomas (negative for PTH, positive for calcitonin, thyroglobulin and TTF1, respectively) metastatic to neck lymph nodes.

7.5 Parathyroid Hyperplasia

7.5.1 Primary Chief Cell Hyperplasia

Parathyroid hyperplasia is defined as an absolute increase in parenchymal cell mass as a result of the proliferation of chief cells, oncocytes and transitional oncocytes, or an admixture, in multiple parathyroid glands [77]. Parathyroid hyperplasia is the cause of primary hyperparathyroidism in about 10–15% of the cases, shows a female/male ratio of 2–3:1 with a wide age range of presentation and highest frequency after the fifth decade [85, 86]. Cystic parathyroid hyperplasia may present clinically as a neck mass [70]. Primary parathyroid chief cell hyperplasia occurs sporadically in about 75% of the cases, while 25% are heritable (Table 7.1). Hyperplastic parathyroid glands have also been documented after neck irradiation and in patients on lithium therapy for psychiatric disorders [77, 80, 81, 85, 87, 89, 98, 133]. Sporadic and familial forms of primary chief cell hyperplasia show indistinguishable histological features, and the terms "chief cell," "chief cell nodular" or "nodular" hyperplasia appear unsatisfactory since both diffuse hyperplastic changes and oncocytic cells may occur. Parathyroid hyperplasia is polyclonal with monoclonality usually restricted to nodular areas [84]. All four parathyroids are usually enlarged, however, due to early diagnosis by serum calcium and family screening the extirpated glands may sometimes show normal histology [85, 86, 133–135].

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 enlargement of glands is symmetric in about 50% of the cases and asymmetric in the rest. 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 [3, 85, 133, 135]. In general, the upper glands are slightly larger than the lower [3], except for MEN 1 parathyroids where the reverse appeared to be true [133]. The nodules can be single or multiple, are more frequent in larger glands, and vary widely in size and cytoarchitectural pattern (Fig. 7.23). 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 hyperplastic and nodular areas may occasionally show fatty tissue resembling normal parathyroid [3, 85, 86, 133, 135] (Fig. 7.23). Nodular and diffuse areas can be composed of chief cells, oxyphil cells, transitional cells, or an admixture. These can be arranged in solid, peripheral palisading, trabecular, cribriform, glandular, papillary, and microcystic patterns, or an admixture (Fig. 7.23). Nuclear pleomorphism and cell multi-nucleation may occur [85, 133, 135]. Glandular structures may look empty, show colloid-like material or contain amyloid [23, 25] which often immunoreact with PTH [133]. Hyperplastic parathyroid tissue with 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 [133] which may be prominent after fine needle biopsy or therapeutic ethanol injection [104].

Chief cells and oxyphil cells are argyrophil and show a polymorphic pattern of expression for chromogranin, PTH, and PTHmRNA [21, 26, 27, 133]. Other neuroendocrine products and cytokeratin have been described in hyperplastic parathyroids [27]. At ultrastructure, hyperplastic cells show

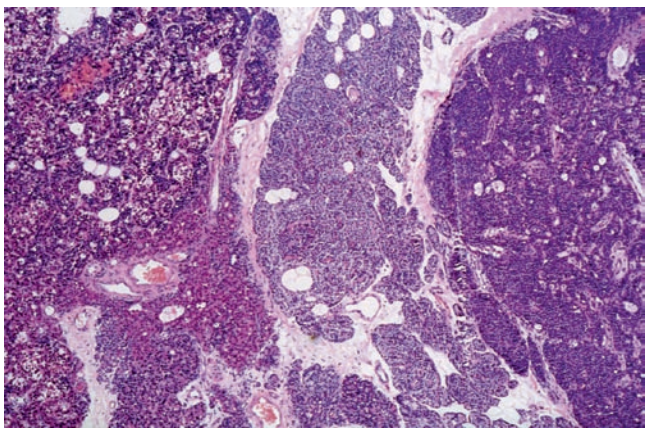

Fig. 7.23 Parathyroid chief cell hyperplasia. Multiple nodules showing cytoarchitectural pleomorphism in a parathyroid from a patient with MEN 1. A few adipose cells are seen admixed with hyperplastic tissue resembling normal parathyroid (H&E, ×20)

features of parathyroid chief cell differentiation, such as membrane bound secretory granules, while oxyphil cells in addition show abundant cytoplasmic mitochondria [20, 135].

Differential diagnoses of primary chief cell hyperplasia include hyperplasia from secondary and tertiary hyperparathyroidism which should be differentiated on clinical grounds due to their similar morphological features. Notwithstanding this, secondary and tertiary hyperparathyroidism show parathyroids with more prominent internodular diffused 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. 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 require immunocytochemistry.

7.5.2 Primary Water-Clear Cell Hyperplasia

Primary water-clear cell hyperplasia causes primary hyperparathyroidism in less than 5% of the cases and shows a wide age range of presentation with the highest frequency in the fifth and sixth decades. While the suggestion that water-clear cells are derived from chief cells is plausible, it is difficult to accept water-clear cell hyperplasia as a variant of primary nodular hyperplasia since 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, 136]. 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 [3, 85, 86, 136]. The disease has been described in supernumerary glands [137] and also in 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 [136].

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, 136, 137]. The glands are chocolate brown in color, lobulated to pseudopodal in shape, and the cut surface is uniform often showing discrete cysts [3, 73]. Histologically, there is diffuse hyperplasia of PTH immunoreactive 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. 7.24). Glandular structures are lined by tall cells with basal nuclei and often contain colloid-like 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 also identifiable on semi-thin sections and secretory granules [20].

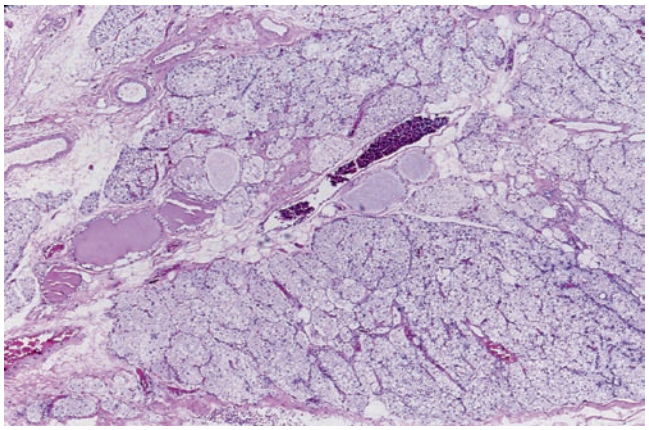

Fig. 7.24 Water-clear cell hyperplasia. Diffuse proliferation of water-clear cells (H&E,×80)

The main differential diagnosis is with water-clear cell adenoma where only one gland is affected. Hyperplasia from secondary and tertiary hyperparathyroidism usually shows nodular changes, oxyphil cells, and chief cells lacking typical water-clear cell histological and ultrastructural features.

7.5.3 Lipohyperplasia

Parathyroid lipohyperplasia is rare and has been described in sporadic form [138], and is occasionally associated with familial benign hipocalciuric hypercalcemia, a disease linked to inactivating mutations of the CaR 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 hyperplastic chief and oxyphil cells. The amount of fat varies from case to case as well as from gland to gland, with a proportion of fat similar to that seen in normal parathyroids [93, 138]. Differential diagnoses include the "solitary" lipoadenoma and primary, secondary and tertiary parathyroid hyperplasia where the fat cell component is absent or scarce in a proper clinical context [133, 139, 140].

7.6 Secondary and Tertiary Hyperparathyroidism

Secondary hyperparathyroidism refers to an adaptive increase of serum PTH due to hypocalcemia that revert to normal if the clinical derangement is brought under control. In tertiary hyperparathyroidism, one or more autonomous tumors (nodules) with non-suppressible 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 [1, 139, 141]. 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 [142].

The classical microscopic picture of secondary hyperparathyroidism is that of a diffuse hyperplasia of chief cells in all parathyroid glands arranged in solid, trabecular, and follicular patterns, or an admixture. The relative fat content is usually low or absent, and the parenchymal cell mass is increased (Fig. 7.25). Oxyphil cells increase in frequency with increasing severity of renal failure, and along with chief cells may form poor to well-defined nodules, making a picture indistinguishable from glands with primary and tertiary hyperparathyroidism [30, 139, 143, 144]. Nuclear DNA content is increased in 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 [145].

Tertiary hyperparathyroidism usually involves all parathyroid glands, although single and double adenomas have occasionally been reported [56, 140]. Ectopic parathyroid glands are found in around one-third of the cases during surgical exploration for tertiary hyperparathyroidism [46, 56]. 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. 7.26), except that the parathyroid glands in tertiary hyperparathyroidism are usually larger and degenerate changes are more common [140, 144]. Large eosinophilic ganglion-like cells have been described in hyperplastic parathyroid tissue from a uremic patient with tuberous sclerosis [146]. PTHmRNA 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 [140].

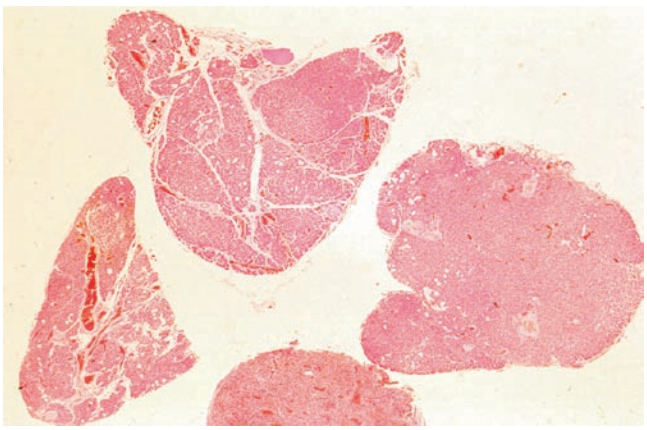

Fig. 7.25 Secondary hyperparathyroidism. Three parathyroid glands from autopsy showing diffuse chief cell hyperplasia with tendency to focal micronodule formation (upper mid) in a patient with chronic renal failure. Lymph node partially seen at mid low (H&E, ×13)

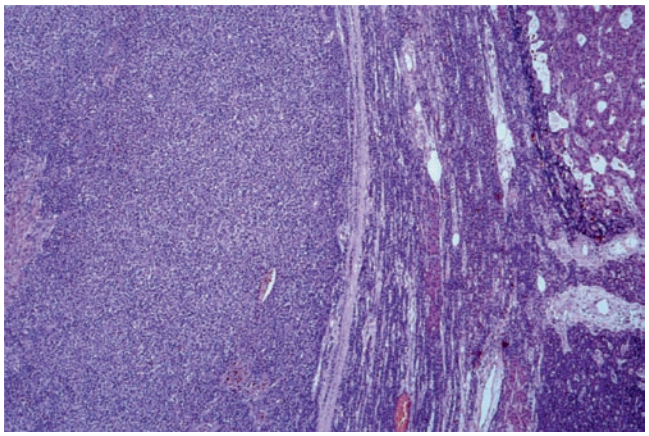

Fig. 7.26 Tertiary hyperparathyroidism. Multiple nodules with cytoarchitectural pleomorphism in a parathyroid gland from a uremic patient. Prominent internodular diffuse oncocytic and chief cell hyperplasia (H&E, ×20)

At ultrastructure, hyperplastic chief cells from both secondary and tertiary hyperparathyroidism often show a reduced number of secretory granules suggesting rapid hormone release [139]. Oxyphil cells are characterized by abundant cytoplasmic mitochondria [30, 139, 144]. Little or no proliferative cell activity is evident from Ki-67 immunocytochemistry [32–34] and the AgNOR technique [35]. 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 among primary, secondary and tertiary parathyroid hyperplasia may not always be possible without knowledge of clinical data, especially in glands incidentally found in post-mortem examination or removal during thyroidectomy or laryngectomy. Secondary and tertiary hyperparathyroidism show parathyroids with more prominent 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.

7.7 Parathyroiditis

Chronic parathyroiditis, an analog to Hashimoto's thyroiditis, is 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, lymphocytes, and sometimes eosinophils admixed with bands of fibrous tissue surrounding residual islands of chief and/or oxyphil cells which may show nuclear pleomorphism (Fig. 7.27). Lymphoid follicles forming germinal centers may occur. Admixed areas of diffuse or nodular parathyroid cell hyperplasia can be present [74, 147–149]. Parathyroid adenomas with hyperparathyroidism and either associated with background massive chronic parathyroiditis [150] or partially replaced by destructive lymphocytic infiltrates resembling chronic parathyroiditis [107] have been described. Mild lymphoid infiltrates can be found in about 10% of normal parathyroid glands at autopsy [149] and, occasionally, in parathyroid hyperplasia [133, 144].

Infectious parathyroiditis caused by *Cytomegalovirus* and *Pneumocystis carinii* have been described in immunocompromised patients due to corticosteroid therapy and AIDS [151, 152]. *Cytomegalovirus* presents histologically as characteristic large purple intranuclear inclusions surrounded by a clear halo in the chief cells. The viral inclusions can be readily identified by immunocytochemistry or in situ hybridization. In *Pneumocystis carinii* infection, the parathyroid parenchyma is replaced in various forms 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. 7.28) and by immunocytochemistry. Disseminated non-caseating granulomas produced by *Mycobacterium tuberculosis* and sarcoidosis have been described in a parathyroid adenoma [153] and in parathyroid tissue [79], respectively.

7.8 Hypoparathyroidism and Other Miscellaneous Disorders

Hypoparathyrodism is a consequence of low PTH secretion leading to hypocalcemia and tetany. The mechanisms leading to hypoparathyroidism can be acquired after removal or destruction of the parathyroid glands or genetically due to defects in parathyroid embryogenesis or the various physiological pathways leading to low serum PTH [154].

The commonest cause of hypoparathyroidism is related to removal of the parathyroid glands during thyroidectomy (0.5–6.6%), parathyroidectomy and radical neck dissection [154]. Hypoparathyroidism has also been described following necrosis and fibrosis of hyperplastic parathyroid glands after percutaneous therapeutic ethanol injection [105].

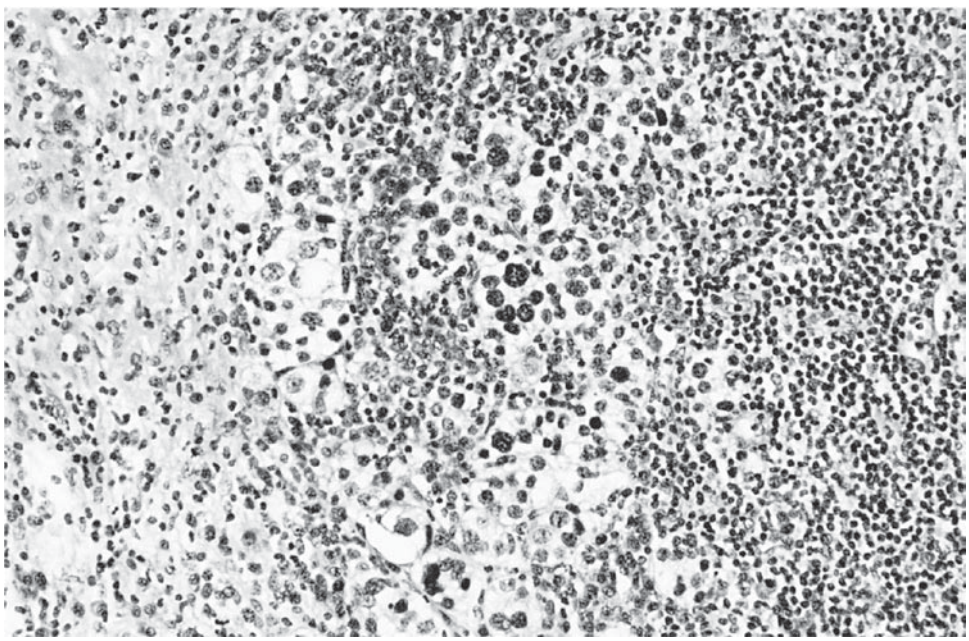

Fig. 7.27 Chronic parathyroiditis. Lymphoid infiltrates admixed with surviving nests of active looking chief cells and oxyphil cells from a 74-year-old male with hyperparathyroidism (H&E, ×360)

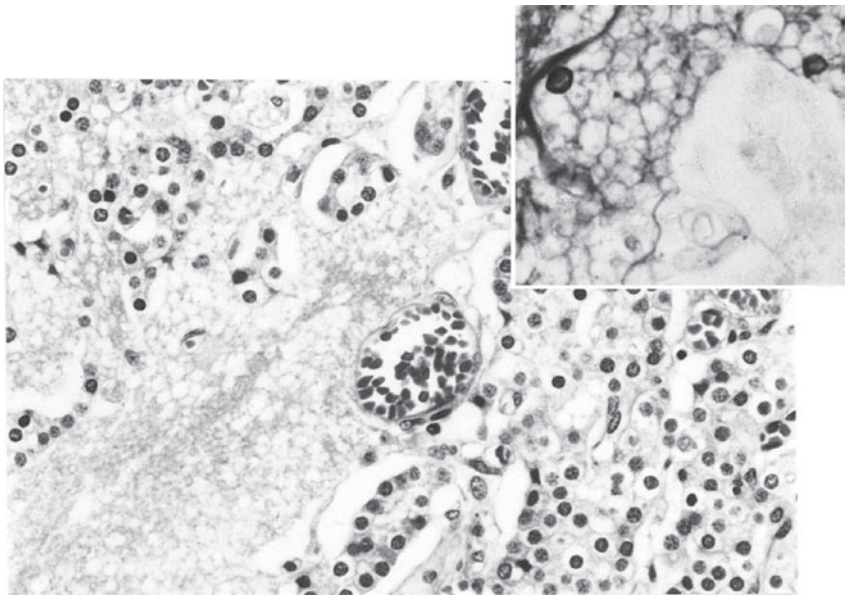

Fig. 7.28 Parathyroid tissue replaced by pink foamy material (H&E, ×360) containing *Pneumocystis carinii* (*inset*: Groccot silver stain, ×360) from a 33-year-old male with AIDS (Courtesy of Professor Sebastian Lucas)

Neonatal hypoparathyroidism is observed in DiGeorge's (or velocardiofacial) syndrome, an autosomal dominant congenital (22q11.2 deletion) developmental anomaly involving the third and fourth pharyngeal pouches characterized by total or partial absence of the thymus and parathyroid glands. It occurs concomitantly with cardiovascular and craniofacial malformations and the patients suffer from severe infections due to thymic hypoplasia or aplasia [154].

Amyloidosis involving all four parathyroid glands may lead to hypoparathyroidism which is rarely a major clinical problem. Amyloid proteins AL (amyloid light chain) and AA (amyloid associated) occur more frequently than expected in the parathyroid glands as these are not usually routinely examined at post-mortem. 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 [155]. Congophilic green birefringent amyloid deposits are observed around the wall of arteries, arterioles, capillaries and, less frequently, within stroma tissue (Fig. 7.29). 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, 155]. Immunocytochemistry for specific amyloid proteins may also help in their distinction.

Progressive systemic sclerosis may produce markedly diffuse parathyroid gland fibrosis and lead to hypoparathyroidism [156] as may also occur in Riedel's thyroiditis with massive fibrous involvement of the parathyroid glands [157].

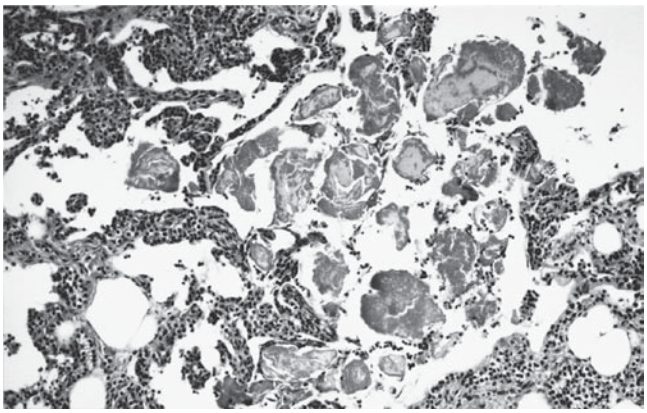

Fig. 7.29 Stromal amyloid deposits in a parathyroid gland from a patient with rheumatoid arthritis (H&E, ×80)

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. 7.30). Hypoparathyroidism occurred when at least 70% of parathyroid tissue from all glands was involved [158].

Hypoparathyroidism has also been described following 131I therapy for hyperthyroidism [159], in association with long-standing iron storage disease, Wilson's disease, and as an autoimmune acquired isolated condition or as part of the hereditary polyglandular syndrome type 1 (mucocutaneous candidiasis, hypoparathyroidism, and adrenocortical failure) [154].

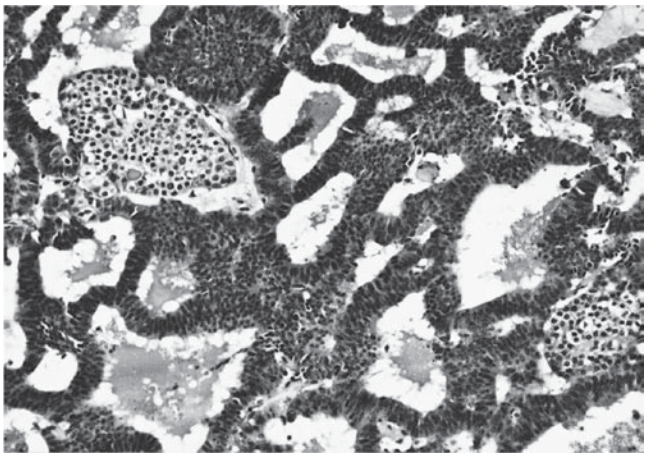

Fig. 7.30 Metastatic bronchial carcinoid to parathyroid gland with chief cell hyperplasia from a 50-year-old male with MEN 1 and hyperparathyroidism (H&E, ×80)

Pseudohypoparathyroidism (Albright's hereditary osteodystrophy) is an autosomal dominant trait manifested by skeletal and phenotypic features associated with hypocalcemia due to PTH resistance linked to inactivating mutation of the Gsα subunit [1, 154]. No histological abnormalities were detected in parathyroid glands from patients with this disorder [160].

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 though hypothyroidism may occur [161].

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 [162].

Parathyroid peliosis [163], hemangioma [164], and Langerhans cell histiocytosis [165] have been described.

7.9 Intraoperative Diagnosis of Parathyroid Disease

Intraoperative frozen section is a useful method for confirming tissue type during the traditional bilateral four gland surgical exploration for hyperparathyroidism. This approach remains relevant in many cases, especially multi-gland disease, and the guidance above should be followed for handling the pathology specimens. Pathologists need to be aware that with recent developments in imaging, some diseased parathyroid glands can be localized pre-operatively, enabling removal by minimally invasive surgery, with or without additional intraoperative techniques other than frozen sections to confirm the removal of all hyperfunctioning parathyroid tissue. This approach is most likely to be used for the most frequent single gland disease (adenoma or carcinoma), and there will be no visualization or sampling of the remaining glands [166]. The major task of the pathologist is to intraoperatively confirm the presence of parathyroid tissue; this can be done either by frozen section or cytological touch preparation. Experience is required for cytological diagnosis which is quick and cost-effective. This method may be useful, particularly for tiny tissues which are difficult to cut, such as a small normal parathyroid gland, small biopsy from a parathyroid lesion, parathyromatosis, and obvious multiple gland enlargement.

The second step is to describe the lesion. Cytological touch preparations can be very helpful in identifying parathyroid proliferative chief or oxyphil 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 [144]. Very rarely, distinct invasion by parathyroid carcinoma can be identified on a frozen section. Parathyroid proliferative lesions with a dominant glandular architectural component 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 which are sometimes present in the thyroid colloid but do not occur in parathyroid glandular structures [58, 59], or to identify birefringent amyloid fibers which 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 of primary hyperparathyroidism, the surgeon will normally look for an ectopic enlarged parathyroid gland, especially in the thyroid, thymus, or carotid sheath. If 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 looking parathyroid gland.

Differential diagnosis rarely influences the intraoperative surgical decision which is based on the number and size of confirmed parathyroid glands found nowadays more frequently by pre-operative imaging techniques. It is not recommended to make a definitive histopathological diagnosis unless obvious.

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Chapter 8
Recent Developments in the Molecular Biology of the Parathyroid

Vânia Nosé and Ashraf Khan

8.1 Molecular Genetics of the Parathyroid Cells

8.1.1 Introduction – Historical Overview

Virchow first described the parathyroid glands in 1863. Ivar Sandström first 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 Vienna, Mandl removed the first parathyroid tumor from a patient with the same disease in 1925.

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 and its function began to be understood during experimental studies of tetanus associated with removal of the glands by F.Albright in 1948. 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]. These two syndromes are, in fact,the same multiple endocrine lesion, which was subsequently recognized by Lulu in 1968 [6]. The preferred designation for this phenomenon is multiple endocrine neoplasia type I (MEN1).

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The parathyroid hormone was amino acid sequenced in 1997, for the first time. Nucleotide sequence of the gene followed in 1990 [7, 8].

The PTH gene was assigned to 11p15, along with insulin, H-ras, and beta-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.--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-kD polypeptide, present also in the kidney, was identified in 1993 [18].

An association with mutations in the human calcium-sensing receptor gene was reported in 1993 in both familial hypocalciuric hypercalcemia (FHH) 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].

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

Hendy et al. [23] reviewed the calcium-sensing receptor mutations in FHH, 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 LOH for chromosome 11 alleles in parathyroid tumors from patients with MEN I [25, 26]. The European Consortium on MEN1 in 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, product of the MEN1 gene, was reported in 1998 to be a nuclear protein [29].

Parafibromin is the 531-amino-acid protein encoded by HRPT2, (1q21-132) a putative tumor suppressor gene recently implicated in the autosomal dominant hyperparathyroidism-jaw tumor (HPT-JT) syndrome [30–32]. Subsequently, somatic mutations have been identified in the majority of sporadic carcinomas. Human parafibromin is a nucleocytoplasmic protein with functions consistent with a tumor suppressor protein [33– 38].

Shattuck et al. [38] first looked for mutations of the HRPT 2 gene, which encodes the parafibromin protein in 21 parathyroid carcinomas from 15 patients who had no known family history of primary hyperparathyroidism or the HPT-JT syndrome, and found that parathyroid carcinomas from 10 of the 15 patients had HRPT 2 mutations. HRPT 2 mutations in the parathyroid carcinomas of three patients were identified as germ-line mutations.

Gill et al. [39] performed immunohistochemistry for parafibromin on 115 parathyroid tissues and found a complete absence of nuclear staining in 3 of 4 (75%) HPT-JT-related tumors and 8 of 11 (73%) sporadic parathyroid carcinomas, and focal weak staining in 1 of 4 HPT-JT-related tumors and 2 of 11 sporadic parathyroid carcinomas. In contrast, 98 of 100 non-HPT-JT-related benign parathyroids showed diffuse strong nuclear positivity, and 2 of 100 showed weak positive staining.

Parafibromin may be used as an additional immunohistochemistry marker for parathyroid tumors [40].

8.1.2 Introduction – 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 parathyroid hormone (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 calcium-sensing receptor (CaR) expressed in the parathyroid cells, C cells of the thyroid, and by the renal cells. In response to decreased serum calcium concentration, CaR on the surface of the parathyroid cells stimulates the release of PTH 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 A2. 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, in the absorption and excretion of calcium and the maintenance of calcium homeostasis (Fig. 8.1).

8.1.3 The Parathyroid Hormone

PTH is synthesized, stored, and secreted primarily by the chief cells of the parathyroid. Initially, it is synthesized as a 115 amino acid-containing precursor, a pre-pro-parathyroid 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 pro-parathyroid hormone, is transported to the Golgi apparatus. There, cleavage of the N-terminus segment occurs, converting the pro-parathyroid hormone to parathyroid hormone. Intact PTH is an 84-amino acid polypeptide with a molecular weight of 9,600. 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 (250HD3) vitamin D to the active form of vitamin D (1, 25

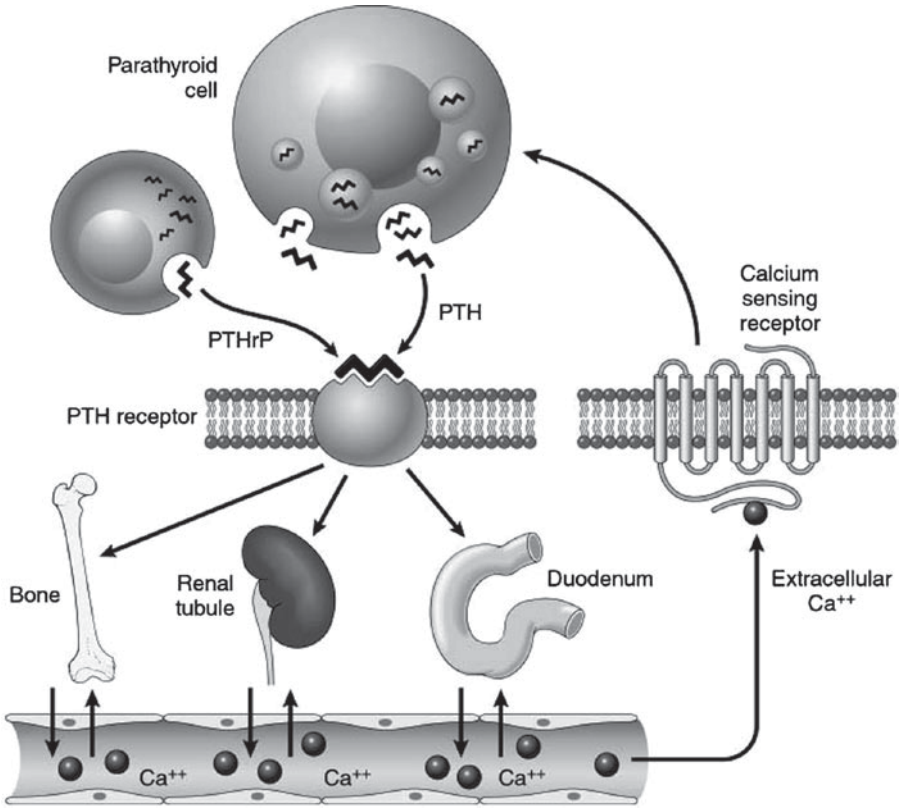

Fig. 8.1 Calcium homeostasis: the parathyroid axis. Activation of the calcium receptor at the parathyroid cell surface leads to increased intracellular calcium, which inhibits PTH secretion. In response to hypocalcemia, there is increase in bone resorption and renal calcium reabsorption, with subsequent increase in calcium levels. 1,25(OH)2D3 increases serum calcium levels by stimulation of intestinal calcium absorption and inhibition of PTH secretion. PTH and PTHrP act by the same receptor (PTH receptor)

dihydroxycholecalciferol). In bone, PTH releases calcium and phosphate into the circulation by bone resorption.

The human PTH gene was mapped to the 11p15 chromosomal band by in situ hybridization, closely linked to beta-hemoglobin gene, the Harvey-ras 1 proto-oncogene, and the insulin gene. Zabel et al. report that 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].

8.1.4 The Parathyroid Hormone-Related Peptide

PTH-related protein (PTHrP) is a homologue of PTH, but is not a true hormone. It shows marked amino-terminal homology to PTH. This amino-terminal end is actively involved in calcium regulation. Serum calcium levels do not regulate the secretion of the PTHrP. Parathyroid hormone 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 weight of approximately 17 kDa, 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 pre-pro-parathyroid hormone was isolated in 1983 [41]. The gene is approximately 4,200 base pairs long and is located at 12p.

8.1.5 Parathyroid Hormone Receptor

The PTHr gene encodes a receptor for both PTH andPTHrP (Fig. 8.1). PTHr is a member of a family of G protein-coupled receptors that includes receptors for secreting 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) [42]. Enchondromas can occur as solitary lesions or as multiple lesions, as in enchondromatosis. A mutant PTH receptor in 2 of 6 patients with Ollier disease was identified [43].

8.1.6 Calcium-Sensing Receptor

A putative calcium-sensing receptor 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. The CaR, whose gene resides on chromosome 3q13.3-21, is a plasma membrane G protein-coupled receptor that is expressed in the parathyroid hormone-producing parathyroid chief cells, renal tubular cells, C cells of the thyroid, bone, and cartilaginous cells. The CaR is a heptahelical molecule, similar to other hormone receptors (Figs. 8.1 and 8.2). 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 calcium-sensing receptor. It is expressed on the parathyroid cell surface and senses fluctuations in the concentration of extracellular calcium [44, 45]. In response to hypocalcemia, PTH elevates serum calcium through enhanced bone resorption and renal calcium reabsorption (Fig. 8.1).

Inactivating mutation in the CaR of the parathyroid glands and the kidneys can cause FHH, 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 calcium receptor 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.

8.1.7 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 negative feedback mechanism inhibiting PTH secretion (Fig. 8.1). The synthesis of osteocalcin, the most abundant protein in bone, is induced by calcitriol, the active hormonal form of vitamin D [46–48].

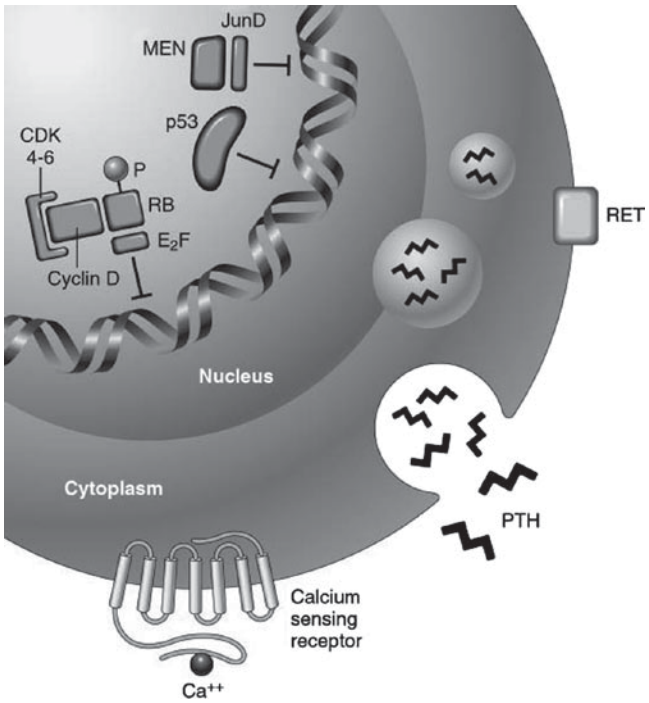

Fig. 8.2 Parathyroid proliferation, gene expression, and secretion. A low serum calcium and/or high serum phosphate lead to a decreased activation of the CaR, with increased PTH secretion. Inactivating mutations in the tumor suppressor-encoded proteins menin, p53, RB, and CaR and activating mutations of the proto-oncoproteins 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

Vitamin D and its metabolites, acting through Vitamin D receptors, decreases the levels of parathyroid hormone 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.

8.1.8 Vitamin D Receptor

Baker et al. [49] 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 (VDR) 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 [50]. The VDR gene contains 11 exons and spans approximately 75 kb.

The VDR gene is located in 12q in humans [51]. This gene was assigned to 12q12-q14 by in situ hybridization [52].

The COL2A1 and VDR loci, both located on chromosome 12q12, are separated by less than 740 kb, with VDR distal to COL2A1 [53]. VDR polymorphism comprises a risk factor for the development of sporadic primary hyperparathyroidism, mostly in females and individuals developing parathyroid adenoma [54]. In MEN1 and uremia, hyperparathyroidism was found to be unrelated to VDR polymorphism [55, 56].

8.1.9 Calcitonin

Human calcitonin contains 32 amino acids and has a molecular weight of 3,421. 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 cholecytokinin. Conversely, a low calcium concentration, dopamine, and alpha 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 [57]. The calcitonin gene is alternatively expressed in a tissue-specific fashion producing either the calcium regulatory hormone calcitonin or calcitonin gene-related peptide [58]. The calcitonin 2 gene produces a second calcitonin gene-related peptide, not a second calcitonin. The pseudogene CALC3 does not encode either peptide. With FISH to prometaphase chromosomes, 2-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 [59, 60].

A human calcitonin receptor cDNA was cloned from an ovarian small cell carcinoma cell line [58]. 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 [58, 61].

8.2 Molecular Genetics of Hyperparathyroidism

8.2.1 Hyperparathyroidism – Introduction

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

Hyperparathyroidism refers to a status of increased production of parathyroid hormone, 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 non-familial 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 non-familial parathyroid adenomas were found to be associated with genetic abnormalities as a pericentromeric inversion on the chromosome 11 (Fig. 8.3). 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 MEN 1 gene. Various chromosomal regions, many loci containing oncogenes, tumor suppressor genes, and gene for calcium-sensing receptors are implicated in the development of some of these tumors. Loss of heterozygozity of 11q13, region containing cyclin D1, MEN 1, and other important oncogenes and tumor suppressor genes (Fig. 8.4) is the most frequent finding in parathyroid tumors, both sporadic adenomas and MEN 1-related parathyroid adenomas [48, 62–74].

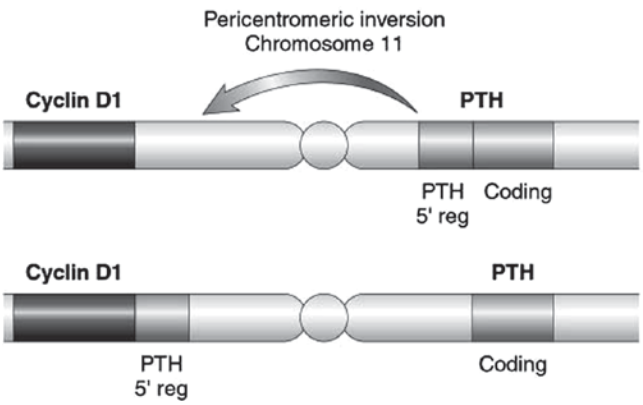

Fig. 8.3 Chromosome 11 Cyclin D1-PTH rearrangement. A pericentromeric inversion of 11p (PTH) and 11q13 (Cyclin D1) places Cyclin D1 under the regulatory region of the gene encoding PTH, causing overexpression of Cyclin D1. The rearrangement separated the PTH gene's 5-prime flanking region from its coding exons

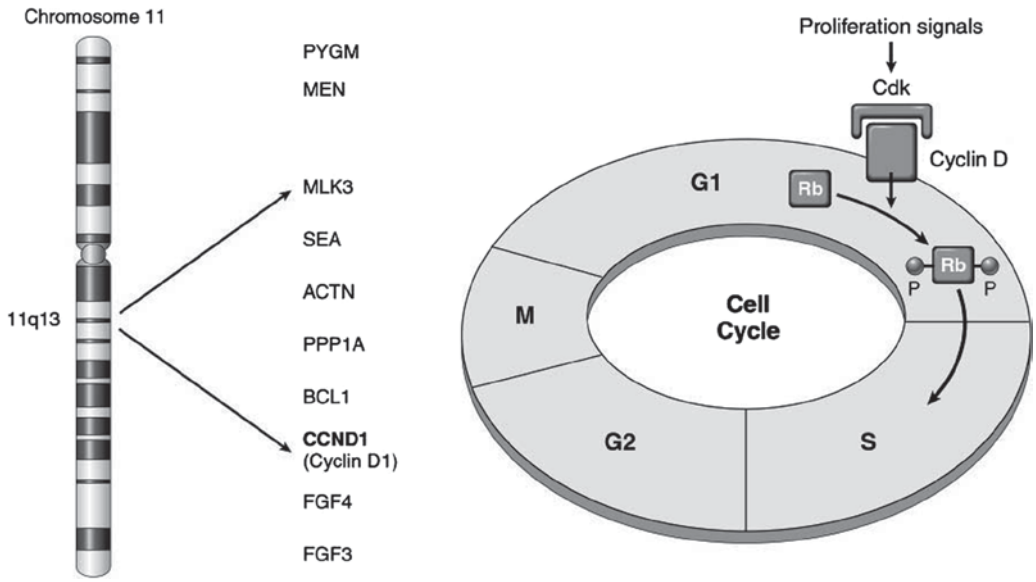

Fig. 8.4 Action of Cyclin D1 and Rb in the cell cycle. Cyclin D1 interacts with Cyclin D-dependent kinases 4 and 6 (Cdk) with hyperphosphorilation of Rb. The inactive Rb allows the cell to enter the G1-S phase of the cell cycle

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 [75–81].

The familial form of hyperparathyroidism is found in many autosomal dominant disorders, among them MEN 1, MEN 2A, hereditary hyperparathyroidism-jaw tumor syndrome, and familial isolated hyperparathyroidism (FIH). Recent genetic information has added to the identification of specific hyperparathyroid diseases, such as neonatal severe hyperparathyroidism, FHH, autosomal dominant mild hyperparathyroidism, and, more recently, familial hypercalcemia and hypercalciuria.

Secondary hyperparathyroidism due to renal failure, hypocalcemia, and hyperphosphatemia leads to increased parathyroid hormone synthesis and secretion, and proliferation of the parathyroid cells (Fig. 8.5). The development of 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 [82, 83].

Hyperparathyroidism has two major findings: calcium-insensitive hypersecretion of parathyroid hormone 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.

8.2.2 Hyperparathyroidism

8.2.2.1 Primary

Sporadic

Hyperparathyroidism can occur in isolated or familial forms. Sporadic primary hyperparathyroidism is mostly of unknown etiology. It is predominant in women over 50 years. External irradiation to the neck is a contributing risk factor for these sporadic forms.

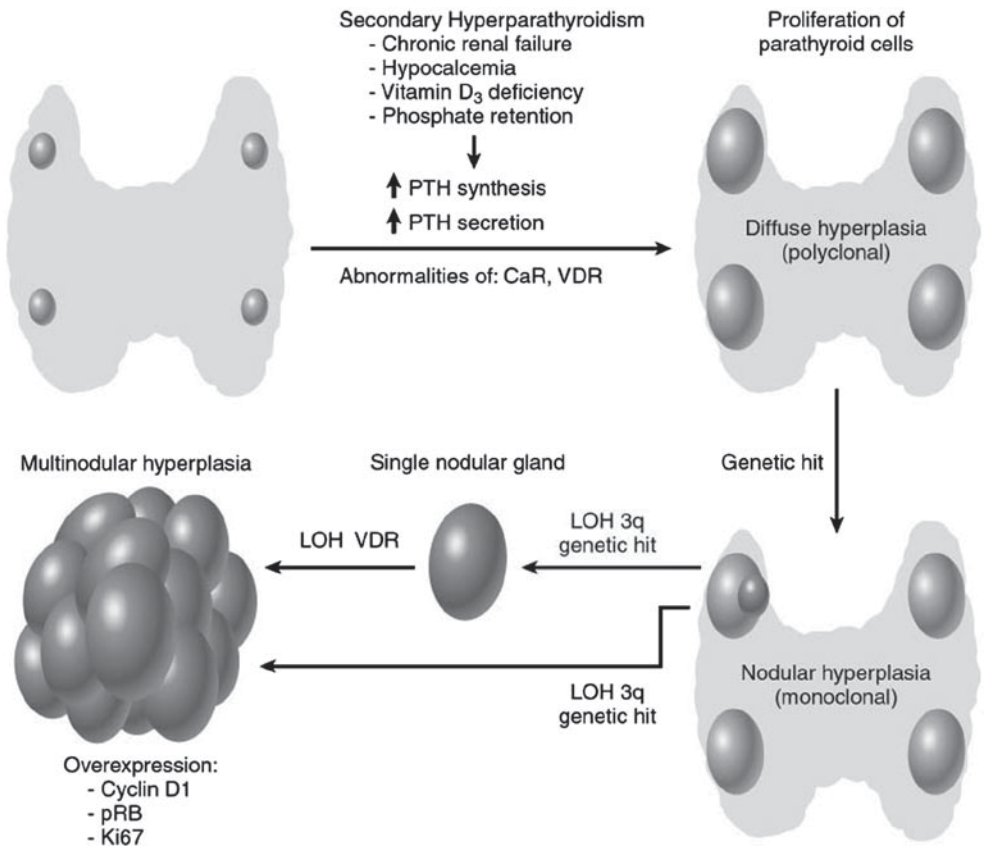

Fig. 8.5 Secondary hyperparathyroidism. Chronic renal failure, hypocalcemia, phosphate retention, and vitamin D deficiency causes diffuse cell proliferation of the parathyroid. The initial parathyroid hyperplasia is polyclonal, and by different genetic hits, it can become monoclonal

Table 8.1 Differential diagnosis of primary hyperparathyroidism

	Inheritance	Number of abnormal glands	Size	Clonality	Surgery
Sporadic adenoma	Not inherited	One, rarely 2	Markedly enlarged (20×)~1.0 g	Monoclonal or oligoclonal	Curative
Multiple endocrine neoplasia type 1 (MEN 1)	Autosomal dominant	Multiple	Enlarged(5–10×) 150 mg – 10.0 g	Monoclonal or oligoclonal	Curative
Multiple endocrine neoplasia type 2A (MEN 2A)	Autosomal dominant	2–3	Enlarged	Not well defined – monoclonal	Curative
Familial hypocalciuric hypercalcemia	Autosomal dominant	Multiple	Normal to mild enlargement	Polyclonal	Not indicated
Neonatal severe hyperparathyroidism	Autosomal recessive	Multiple	Very enlarged	Polyclonal	Total parathyroidectomy
Hyperparathyroidism-jaw tumor syndrome	Autosomal dominant	Multiple with cysts	Asynchronously enlarged;Higher incidence of cystic carcinoma	Monoclonal	"Curative"
Familial isolated hyperparathyroidism	Autosomal dominant	Single or multiple	Higher incidence of carcinoma	Monoclonal or oligoclonal	Curative
Sporadic carcinoma	Not inherited	One	Markedly enlarged	Monoclonal	"Curative"

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 approximately 15–20%, with carcinomas accounting for rare cases. Some cases are part of inherited syndromes (Table 8.1).

Solitary parathyroid adenomas or multiglandular hyperparathyroidism is monoclonal or oligoclonal proliferation.

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. 8.4), is deleted in a third of sporadic primary hyperparathyroidism. In approximately half of these cases, a somatic mutation of MEN1 has been identified [48, 62, 66, 81, 84–89]. 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; lymphomas and only few epithelial cell tumors have been correlated with specific chromosomal rearrangements with resulting fusion proteins that are demonstrated to contribute to the neoplastic process. In parathyroid lesions the pericentromeric inversion of 11p15 and 11q13, placing the cyclin D1 under the regulatory region of the gene encoding parathyroid hormone, is found in a minority of tumors. A high frequency of LOH at 1p and 11q in tumors of primary hyperparathyroidism suggests that inactivation of MEN1 gene and putative tumor suppressor genes at these regions are 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. 8.6), 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.

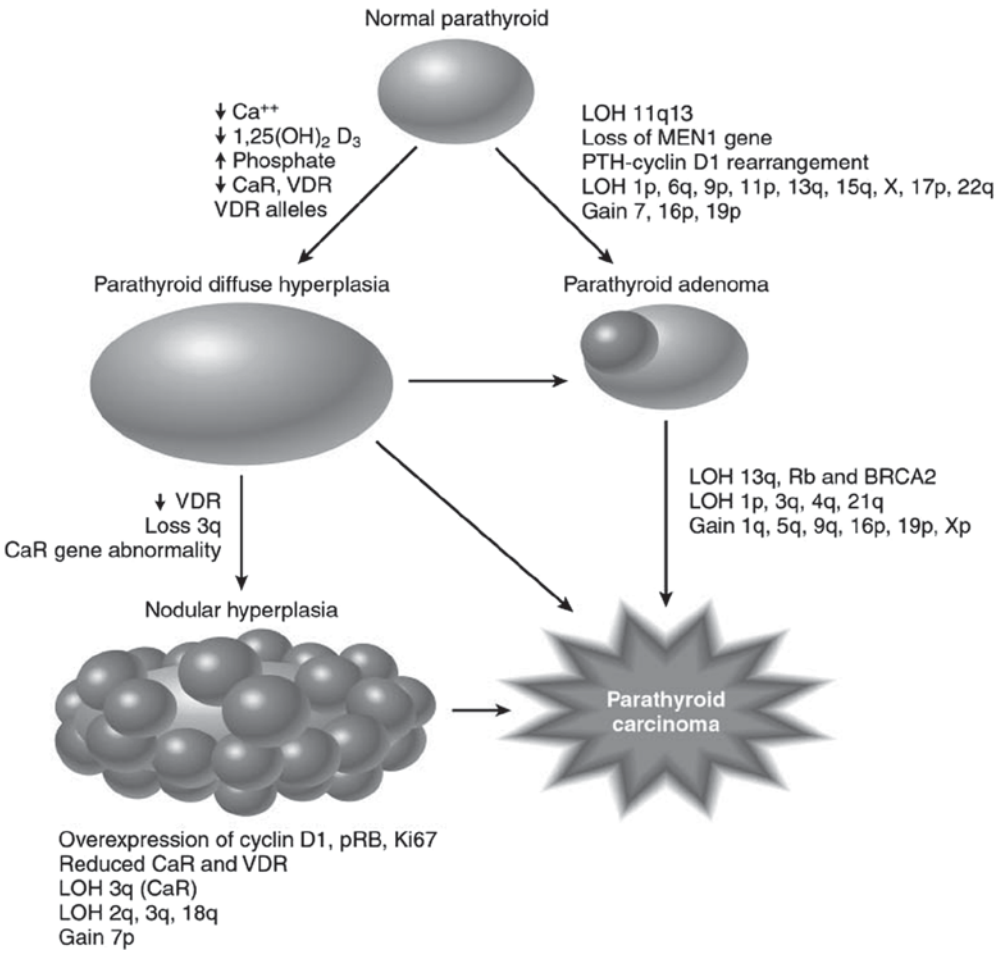

Fig. 8.6 Proposed model of development of the diverse parathyroid disease. Proliferation-promoting factors lead the normal parathyroid to proliferate, and can lead to disease progression. The polyclonal parathyroid hyperplasia can lead to a monoclonal proliferation by acquired somatic mutations, including some losses or gains of chromosomal regions

The findings associated with this disorder are more commonly 11q13 loss, somatic mutation of MEN1 gene, and reduced calcium-sensing receptor, with loss of 3q. There is overexpression of Cyclin D1, and retinoblastoma protein [88, 90–93].

Vitamin D plays a crucial role in the regulation of the parathyroid glands. Vitamin D receptors (VDR) A, B, and T alleles are over-represented in primary hyperparathyroidism, particularly in postmenopausal female patients [54, 94].

Parathyroid Adenoma. Parathyroid adenomas are common. They account for approximately 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 upon 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 loci, 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 multiple endocrine neoplasia type 1 (MEN1), some lesions being classified as sporadic primary parathyroid hyperplasia and most pathological parathyroid glands excised at surgery for secondary hyperparathyroidism, as a result of renal and parathyroid carcinomas [26, 68, 93, 95–100]. 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 parathyroid hormone 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 parathyroid hormone gene promoter drove a translocated sequence encoding cyclin D1, at 11p15 and 11q13, which indicated a PTH-cyclin D1 gene rearrangement, and overexpression of cyclin D1 (Fig. 8.3).

Cyclin D1 is important for the entry of proliferating cells into G1 phase of the cell cycle. D-cyclins regulate the G1 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 G1 into S phase (Fig. 8.4). 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 calcium-sensing receptor (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 [63].

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 [87]. However, there is no pathogenic importance in the development of primary hyperparathyroidism [87].

Multiple endocrine neoplasia type 1 (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. LOH 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% [86, 101]. 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 [86].

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 chromosomes 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 (MTC) [102, 104]. Another more recent study reveals that inactivation of tumor-suppressor genes in 1p seems to be an independent phenomenon of inactivation of tumor-suppressor genes in 11q, since allelic

losses in chromosome 1p are not associated with allelic losses in 11q [105]. 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 [106].

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 in 16p (11%) and 19p (9%) were shown in many patients with sporadic parathyroid adenomas by comparative genomic hybridization, suggesting possible oncogenes in chromosomes 16p and 19q [107]. Frequent losses of heterozygosity were reported in chromosomes 1p (27%), 6q (19%), 11p (29%), 11q (31%), and 15q (28%) 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 in these chromosomal arms (Table 8.3).

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 8.5).

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 MEN-1 gene product menin has been identified, and mutations contribute to sporadic tumors. Mutations in the RET gene (MEN-2) contribute rarely to development of sporadic parathyroid tumors. Mutations in the CaR gene play a role in familial disease, but 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 [108]. The etiology of parathyroid carcinoma is unclear, although there is a relationship with having a 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 twelve end-stage renal disease patients, who between 1982 and 1996 were receiving maintenance hemodialysis [109], demonstrated hyperplasia of other parathyroid glands and one had a history of prior neck irradiation [110].

Carcinoma has been reported in association with familial hyperparathyroidism particularly in the autosomal dominant form with isolated hyperparathyroidism that is not part of the multiple endocrine neoplasia type I (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 pericentric inversion in chromosome 9 [111]. There was no evidence of ras gene mutations, PTH gene arrangement, or allelic loss from chromosome 11q13 in one patient studied.

Table 8.2 Familial syndromes associated with parathyroid disease

Syndrome	Clinical features	Chromosome location	Gene/protein	Inheritance
Multiple endocrine neoplasia type 1 (MEN 1)	Multiglandular parathyroid hyperplasia (90%) Gastroenteropancreatic (60%) Pituitary tumors (30%)	11q13	MEN1/menin	Dominant
Multiple endocrine neoplasia type 2A (MEN 2)	C-cell hyperplasia/neoplasia (100%) Pheochromocytomas (30%) Parathyroid hyperplasia (20–30%)	10q11.2	RET	Dominant
Familial hypocalciuric hypercalcemia	Hypercalcemia with primary Parathyroid hyperplasia and inappropriate serum PTH	3q13-q21 19p13.3 19q13	CaSR	Dominant
Neonatal severe hyperparathyroidism	Marked parathyroid hyperplasia, symmetric, diffuse Due to calcium insensitivity	3q13-q21	CaSR	Dominant
Hereditary hyperparathyroidism-jaw tumor syndrome	Solitary parathyroid cystic adenoma/ cystic carcinoma Fibro-osseous jaw lesions Wilms Polycystic kidney disease renal hamartomas	1q21-q32	HRPT2/ parafibromin	Dominant
Familial isolated hyperparathyroidism	Solitary or multiple Parathyroid disease Multiglandular hyperplasia Hypercalcemia	1q21-q32 11q13 3q13.3	HRPT2MEN1 CaSR	Dominant
Autosomal dominant mild hyperparathyroidism	Parathyroid hyperplasia/adenoma Hypercalcemia Hypercalciuria	3q13-q21	CaSR	Dominant

Table 8.3 Molecular differential diagnosis of hyperparathyroidism

Cause	Findings
Primary	
Adenoma, sporadic	LOH MEN1 gene, (25–40%); homozygous mutations of MEN1 gene (15–20%)
	CCND1/PRAD1 translocation (<5%)
	Cyclin D1 overexpression CCND1 amplification (20–40%)
	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
MEN 1	A.D., germline mutation 11q13, MEN1
MEN 2A	A.D., germline mutation 10q21, RET
Familial hypocalciuric hypercalcemia	A.D., 3q13.3-q21 (CaR)
	19p13.3 (?gene)
	19q13 (?gene)
Familial hypercalcemia	A.D., 3q13.3-q21(CaR)
Neonatal severe hyperparathyroidism	A.R., 3q13.3-q21 (CaR)
Hyperparathyroidism-jaw tumor	A.D., 1q21-q23 (HRPT2 locus)
	LOH 1q, 1p, 11q13
Familial isolated hyperparathyroidism	A.D., 1q21-q23 (HPT-JT or HRPT2 locus)
	MEN1, 11q13
Carcinoma	LOH 13q with loss BRCA2
	LOH 13q and 1p in 40% with loss of Rb
	Mutation of HRPT-2 gene (1q1-q 23)
	LOH of P53, abnormal expression of p53 protein
	Gains 1q, 5q, 9q, 16p, 19p, Xp
	LOH of 1p, 3q, 4q, 6q, 9p, 13q, 21q
Primary hyperplasia	LOH 11q13, somatic mutation MEN1
	Reduced CaS receptor, with loss 3q
	Reduced vitamin D receptors
	Overexpression pRb, CyclinD1, Ki67
Autosomal dominant mild hyperparathyroidism	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 CaR reduced in nodular hyperplasia
Chronic renal failure	CaR gene abnormality (LOH 3q in 10%)
Malabsorption	Reduced vitamin D receptors
Vitamin D deficiency	
Renal tubular acidosis	

Table 8.4 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-p1322q11.2-pter
Late neonatal hypocalcemia: Isolated or part of Kearns-Sayre, Kenny-Caffey syndromes	
Autosomal dominant hypoparathyroidism/hypocalcemia	Activating mutations of CaR gene
Sporadic idiopathic hypoparathyroidism	Activating mutations of CaR gene
FamilialIsolated familial 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
Pseudohypoparathyroidism type 1	Gs-alpha one protein of the adenylyl cyclase complex (GNAS1) mutation
–Pseudohypoparathyroidism type 1a: Albright's hereditary osteodystrophy	Mutation in the stimulatory GNAS1
–Pseudohypoparathyroidism type 1b	?Mutations in the GNAS1-imprinting defect of GNAS1
Pseudohypoparathyroidism type 2	Defective cyclic AMP-dependent protein kinase
Autoimmune polyglandular syndrome type I	Autoantigen to CaR

Table 8.5 Genes involved in parathyroid diseases

Gene/location	Protein	Function/defect	Lesion	Evidence
MEN1-11q13	Menin	Tumor suppressor	Hyperplasia	LOH in adenomas
			Adenoma	MEN mutations
Cyclin D1-11q13	Cyclin D1	Cell cycle regulation	Adenoma	Rearrangement 11q13 and 11p15
			Carcinoma	Amplification of cyclin
			Secondary HPT	LOH in secondary HPT
CaR-3q13.3-q21	Calcium receptor	Calcium-sensing receptor	Hyperplasia	LOH in adenomas and secondary HPT
			Adenoma	
			Familial – hypocalciuric – hypercalcemia	One mutation: Familial hypocalciuric hypercalcemia
			Neonatal severe HPT	Two inactivating mutations: Neonatal severe HPT
Rb-13q14	Rb	Tumor suppressor	Carcinoma	LOH Rb in carcinomas
		Cell cycle regulation		Immunoreactive Rb detectable
RET-10q11.2	RET tyrosine kinase	Oncogene	Hyperplasia	Somatic mutation RET
			Adenoma	
HRPT2 gene-1q21-q32	Parafibromin	Tumor suppressor	Familial Isolated HPT	LOH in adenomas
			HPT-JT	LOH in familial isolated HPT, HPT-JT, carcinoma – mutation in carcinoma
			Carcinoma	
?11q23	Unknown	Tumor suppressor	Adenoma	LOH 11q23 is frequent in adenomas
P53-17p13.1	P53	Tumor suppressor	Carcinoma	LOH p53
			Adenoma	Point mutation p53
?1p35-36	Unknown	Tumor suppressor	Adenoma	LOH 1p35-36 frequent
Vitamin D receptor			Adenoma	Polymorphisms
BRCA2		Tumor suppressor	Familial isolated HPT	LOH 13q in familial isolated HPT
13q				
1q, 5q, 9q, 16p, 19p	Unknown	Oncogene	Carcinoma	Gains in carcinoma
1p, 3q, 4q, 13q, 21q	Unknown	Tumor suppressor	Carcinoma	–Losses in carcinoma
6q, 9p, 15q	Unknown			

In addition, a greatly increased risk of parathyroid carcinoma is associated with the hereditary hyperparathyroidism-jaw tumor syndrome, related to 1q21-q31 [112, 114].

Familial hyperparathyroidism and parathyroid carcinoma are rare, and further studies should clarify their relationship. A case of parathyroid carcinoma in an 8-year-old girl whose mother had previously undergone parathyroidectomy for primary hyperparathyroidism suggests that it may have a familial basis [115].

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 [88] and in two of three in another [89]. 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 fluorescent in situ hybridization [116]. All carcinomas demonstrated overexpression of cyclin D1 by immunohistochemistry (Figs. 8.7 and 8.8). Using FISH in paraffin sections, confirmation of polysomies of chromosome 11 and cyclin D1 was possible in the neoplastic cells (Fig. 8.8). 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 [117]. Strong evidence exists for the presence of a gene on chromosome 13q whose acquired inactivation contributes to the development of parathyroid carcinoma (Table 8.3). Parathyroid carcinomas were investigated for evidence of loss of a region in chromosome 13 containing Rb and for altered expression of Rb protein [79]. All 11 parathyroid malignancies lacked a Rb allele, and most had complete absence of nuclear staining for the Rb protein. Allelic loss of Rb or D13S71 at 13q14 in a parathyroid carcinoma has also been reported [80]. Loss of 13q is found frequently in parathyroid carcinomas [118].

An allelic deletion of the 13q12-14 region also involves the hereditary breast cancer susceptibility gene (BRCA2). It

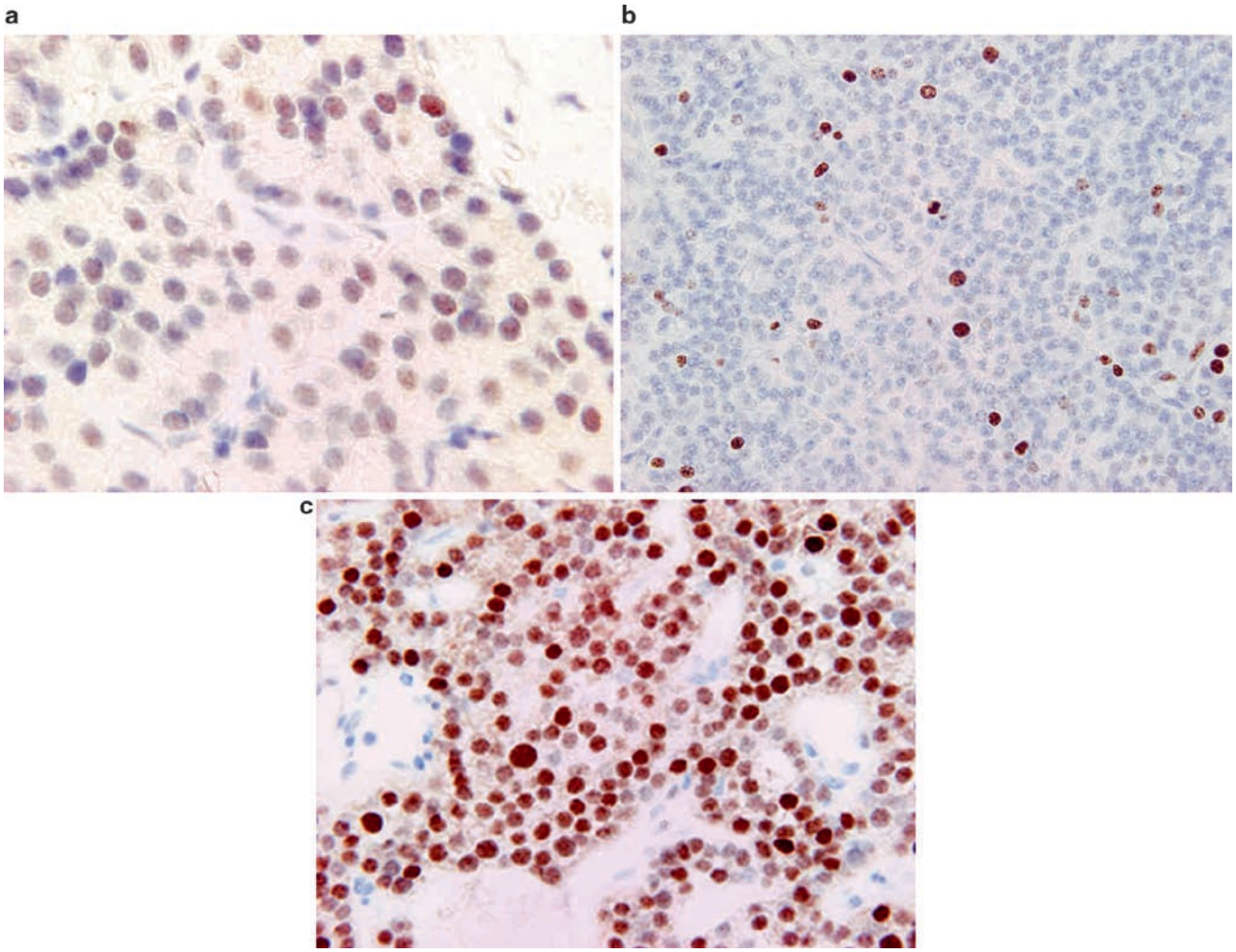

Fig. 8.7 Immunohistochemistry for p53, Ki-67, and Cyclin D1 in parathyroid carcinoma. P53 was observed in parathyroid carcinoma (Fig. 8.7a). There is a higher proliferative activity index, demonstrated by Ki-67 immunostain in parathyroid carcinomas compared with parathyroid adenoma and hyperplasia (Fig. 8.7b). Parathyroid carcinoma showing overexpression of Cyclin D1 (Fig. 8.7c)

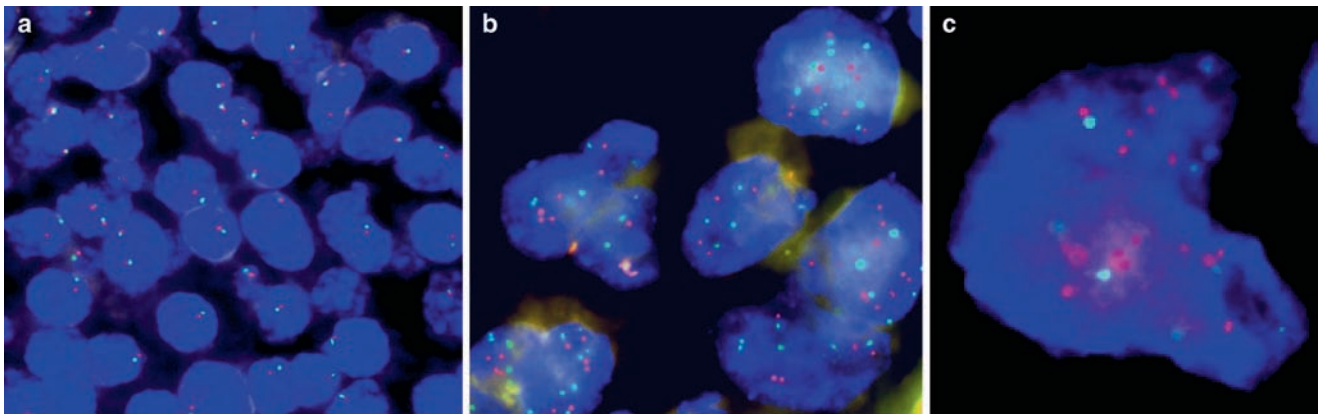

Fig. 8.8 FISH for Chromosome 11 and Cyclin D1 in parathyroid adenoma and carcinoma. Paraffin-embedded sections of parathyroid neoplasms demonstrating up to two copies of chromosome 11 and Cyclin D1 in adenomas (up to two red and green signals) (Fig. 8.8a). Primary and metastatic carcinoma showing polysomies of chromosome 11 and Cyclin D1 (multiple red and green signals) (Figs. 8.8b and 8c)

was found in 3 of 19 parathyroid adenomas, which had aggressive features, and 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 [77].

This 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. 8.7) is low [75, 78]. 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 [118, 119]. Several recurrent abnormalities that seem to be preferentially or exclusively appearing in carcinomas, compared with adenomas, were found in some studies [80, 103, 118, 119]. 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 8.3). The regions commonly lost in adenomas, such as 11q13, were rarely lost in carcinomas [108, 115, 116, 118, 119].

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.

One of the most important advances in the molecular pathology of parathyroid carcinoma was recently identified with the isolation of the genetic mutations in hyperparathyroidism – jaw tumor syndrome (HPT-JT). Patients with this syndrome have a combination of tumors that include parathyroid cystic lesions, as well as adenomas and carcinomas, fibro-osseous lesions of the jaw, and other rare neoplasms [30, 35, 38–40]. The gene involved in the hyperparathyroidism-jaw tumor syndrome is the HRPT2 gene in chromosome 1q21-q31.2, which encodes a 531-amino-acid protein, parafibromin [35, 38].

Parathyroid carcinomas from patients with sporadic settings, as well as those with HPT-JT syndrome, frequently have a LOH of the HRPT2 gene, with a loss of immunoexpression of parafibromin protein product [30, 35, 39, 40].

Mutation of the HRPT2 gene was found in sporadic parathyroid carcinoma from 10 of 15 patients. Shattuck et al. [38] first looked for mutations of the HRPT 2 gene, which encodes the parafibromin protein in sporadic parathyroid carcinoma as germ-line inactivating HRPT 2 mutations have been found in familial hyperparathyroidism – hyperparathyroidism-jaw tumor syndrome, which carries an increased risk of parathyroid carcinoma. They did direct sequencing of the full coding, and flanking splice-junctional regions of the HRPT 2 gene in 21 parathyroid carcinomas from 15 patients who had no known family history of primary hyperparathyroidism or the HPT-JT syndrome, and found that parathyroid carcinomas from 10 of the 15 patients had HRPT 2 mutations, all of which were predicted to inactivate the encoded parafibromin protein; HRPT 2 mutations in the parathyroid carcinomas of three patients were identified as germ-line mutations.

These findings suggest that patients with HRPT2 gene mutations (1q 21-q 32) have HPT-JT syndrome or a variant of this syndrome, as a subset of patients with mutation-positive carcinomas have germline mutations for the HRPT2 gene.

Mutation analysis of the HRPT2 gene [120] was undertaken in five HRPT2, three FIHP, three MEI1, one MEN2A, 25 sporadic adenomas, 17 hyperplastic parathyroid glands, two lithium-associated tumors, and four sporadic carcinomas. All the four cases of carcinoma were detected with HRPT2 somatic mutations. Germline mutations were found in all five HPT-JT parathyroid tumors, and in two of three FIHP cases. These findings support the role of HRPT2 as a tumor suppressor gene in sporadic thyroid carcinomas, and HRPT2 as the causative gene in HPT-JT and FIHP.

Gill et al. [39] performed immunohistochemistry for parafibromin on 115 parathyroid tissues comprising four HPT-JT related tumors, 11 sporadic parathyroid carcinomas, 79 sporadic adenomas, three multiple endocrine neoplasia, 2A-related adenomas, two sporadic primary hyperplasias, two multiple endocrine neoplasia-1-related hyperplasias, six secondary hyperplasias, four tertiary hyperplasias, and four normal parathyroid glands. There was complete absence of nuclear staining in three of four (75%) HPT-JT related tumors, and eight of 11 (73%) sporadic parathyroid carcinomas, and focal weak staining in one of four HPT-JT tumors and two of 11 sporadic parathyroid carcinomas. Only one parathyroid carcinoma exhibited diffuse strong nuclear expression of parafibromin. In contrast, 98 of 100 non-HPT-JT related benign parathyroids showed diffuse strong nuclear positivity and two of 100 showed weak positive staining. They concluded that complete absence of nuclear staining for parafibromin is diagnostic of parathyroid carcinoma or an HPT-JT-related tumor [40, 121–124].

Recent reviews on the subject suggest parafibromin as an immunohistochemistry marker for carcinoma, although a subset of parathyroid adenoma cases have also shown loss of parafibromin expression [125].

Familial Hyperparathyroidism

Familial hyperparathyroidism is usually part of multiple endocrine lesions, and occurs in families without evidence of other endocrine diseases. The diagnosis of chief cell hyperplasia

should be followed by the study of other organ involvement in the patient and in the family.

Multiple endocrine neoplasia (MEN) is characterized by involvement of two or more endocrine glands with neoplasia. These syndromes are rare. There are two major forms, referred to as MEN 1 and MEN 2. Parathyroid, 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 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 other inherited familial disorders have been reported. The knowledge of tumorigenesis in inherited parathyroid disease led to the description of hyperparathyroidism-jaw tumor syndrome, FHH, neonatal severe hyperparathyroidism, FIH, and more recently, autosomal dominant mild hyperparathyroidism (Tables 8.1–8.3).

Multiple Endocrine Neoplasia 1 (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 non-endocrine and endocrine neoplasia. The MEN 1 gene is located in chromosome 11q13 (Fig. 8.4). It is a tumor suppressor gene. Patients affected by familial MEN1 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 MEN1 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. 8.2) [29]. The interaction between JunD and Menin has yet to be elucidated [126].

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 with 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.

Multiple endocrine neoplasia type 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 the patients. Prolactinomas are the commonest pituitary tumor, and are present in 50% of MEN 1 patients [25, 64, 127–129].

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 [65].

Adrenal cortical tumors are common in MEN 1. Smooth muscle tumors, as well as thyroid follicular neoplasms have not been confirmed as being MEN 1 related [130]. Pheochromocytomas are rare in MEN1.

As in the familial MEN1-associated tumors, sporadic tumors may develop as a result of MEN germline mutations associated with somatic LOH loss at 11q13. Somatic mutations in sporadic parathyroid adenomas have been reported as high as 22% [73, 84, 131, 132]. Other sporadic tumors associated with these mutations are gastrinomas, insulinomas, lipomas, angiofibromas, vasoactive intestinal peptide-secreting tumors, and lung and gastric carcinoids.

Multiple Endocrine Neoplasia 2A (MEN 2A). Multiple endocrine neoplasia type 2A or Sipple syndrome is an autosomal dominant disease. This disorder is characterized by primary parathyroid hyperplasia and hyperparathyroidism, pheochromocytoma, and C-cell hyperplasia/MTC. The hyperparathyroidism is caused by multiglandular parathyroid hyperplasia or adenoma, and is present in about 20% of cases. MTC, however, is seen in almost all patients with inherited MEN 2A gene [133].

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 are involved in cell growth and differentiation. Some versions of the rearrangement of the proto-oncogene RET, called RET/PTC are specific markers of papillary thyroid carcinoma. This rearrangement leads to the formation of chimeric oncogenes. Similar rearrangements have not been identified in parathyroid lesions.

The germline mutation of the RET in MEN 2A results in a gain of function, different from other inherited diseases. Those diseases are a 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 (condon 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 of condon 634 (condon 11), Cys to Arg, is found preferentially in families of MEN 2 syndrome with hyperparathyroidism, [133] and is the most common genetic finding in this disease. The penetrance of hyperparathyroidism in the patients with mutation of condon 634 is about 20%. RET

mutations have not been identified in sporadic parathyroid adenomas. Mutations in exon 13 of the RET (condons 790 and 791) occur less often [74]. Mutations in condon 768 of exon 13 are 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. Jackson first reported a family with hereditary hyperparathyroidism associated with jaw tumors [134]. 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 [113].

The hyperparathyroidism and jaw tumor (HPT-JT) syndrome is an autosomal dominant familial disorder, linked to the chromosomal region of 1q32-q21 (HRPT2 locus) [135–137]. There were 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) [138].

There is no clear association 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 [139]. Some authors found no association of changes in 1q, 11q, and X in the cases of HPT-JT in one family [140].

Thirteen affected members of a family presented with either parathyroid adenoma or carcinoma. In 5 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 Hürthle cell adenoma of the thyroid, excluding the involvement of the MEN1 gene [72].

There is a suggestion that cystic parathyroid tumors might represent a different subgroup among parathyroid neoplasia.

Familial Isolated Hyperparathyroidism. 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 multiple endocrine neoplasia 1; there is a tendency towards malignant transformation in the glands. Whether FIH is a variant of multiple endocrine neoplasia type 1 or HPT-JT syndrome is yet to be established [114, 131, 141].

In parathyroid tumors from FIH families, repeated allelic losses of the HRPT2 region in 1q21-32 were reported [62, 135].

Some authors found no MEN1 germline mutation [132]. However, another cause of FIH is mutation in the MEN1 gene [131, 142, 143].

Analysis of tumor DNA from four patients from a single family with FIH, showed limited LOH on 9p22-p21 and 13q12.3-q32 [76]. The authors suggested a possible contribution of tumor suppressor genes, such as retinoblastoma gene and the hereditary breast cancer susceptibility gene (BRCA2) in 13q as being associated with the parathyroid tumors in this family.

Screening for other tumors associated with MEN1 and HPT-JT should be performed for the diagnosis of FIH. More genetic studies of FIH families are needed to better clarify the genetic basis of this disease.

Familial Hypocalciuric Hypercalcemia. Hypercalcemia, hypercalciuria, and familial benign 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 8.1) [144]. FHH prevails in about half of the cases of hypercalcemia during the first two decades of life.

Loss-of-function mutations in the CaR gene are responsible for this disease [19, 145], as well as for neonatal severe hyperparathyroidism. Gain-of-function mutations result in autosomal dominant hypocalcemia, discussed later [145–149].

A form of autosomal dominant hypoparathyroidism linked to a region of 3q13 suggests that it might be caused by an inactivating mutation in the CaR, with suppression of PTH secretion and lowering of the set point for serum calcium levels [150].

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 is caused by loss-of-function mutations in the calcium-sensing gene (CaR) [23, 148]. Some authors have demonstrated that heterozygous mutations

in the calcium-sensing receptor can also cause the disease [19, 151]. The authors conclude that dosage of the gene defect accounts for the different phenotypes; a single defective allele causes FHH, while two defective alleles cause 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 studies under progress are helping and will continue to help our understanding of tumorigenesis. Autosomal dominant inheritance is found in several inherited disorders as MEN1, MEN 2A, hereditary hyperparathyroidism-jaw tumor syndrome, and FIH (Table 8.1).

New phenotypes, different from previously known disorders, have been reported, as autosomal dominant mild hyperparathyroidism [152]. This is caused by a mutation in the cytoplasmic tail of the calcium receptor. Autosomal dominant mild hyperparathyroidism is rare, and is 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 are associated with the development of parathyroid neoplasia. Familial hypercalcemia and hypercalciuria have been described, as being associated with parathyroid hyperplasia or adenoma. This rare disorder is associated with mutations of the CaR (Tables 8.1–8.3).

8.2.2.2 Secondary and Tertiary Hyperparathyroidism

Chronic renal failure, hypocalcemia, active vitamin D deficiency, and phosphate retention, all stimulate the synthesis and secretion of parathyroid hormone and proliferation of parathyroid cells (Fig. 8.5). Reduction of vitamin D receptors (VDR) and reduction of calcium sensing receptors (CaR) also contribute to secondary hyperparathyroidism [48, 63, 82, 152]. 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 G0 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. Secondary hyperparathyroidism is a monoclonal process.

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 calcium-sensing receptor expressed in the parathyroid cells, the C cells of the thyroid, and by the renal cells. Thus, the CaR is activated by an 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. Phosphate's effect on the parathyroid is mediated by phospholipase A2. Parathyroid cell proliferation is increased by chronic hyperphosphatemia and dramatically decreased by hypophosphatemia.

Over-expression of Cyclin D1 was not seen in secondary parathyroid hyperplasia by some authors [81, 100], while it was found in 40% of primary adenomas. Nodular hyperplasia showed greater expression of cyclin D1, pRb, and Ki67 when these cases were compared with diffuse hyperplasia (Fig. 8.6). A higher prevalence of p53 expression in secondary hyperparathyroidism suggests that p53 might be involved in some of these cases [75].

The CaR was seen reduced in nodular hyperplasia as compared with that in diffuse hyperplasia, in both mRNA and protein concentrations [153].

Some parathyroid glands from patients with uremic refractory secondary/tertiary hyperparathyroidism show monoclonal neoplasms. A large group of uremia-associated parathyroid tumors were studied to determine the genetic abnormalities that underlie clonal expansion of this disorder. By 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 (2%); the tumor also contained a somatic mutation of the remaining MEN1. A total of 13% of tumors demonstrated recurrent allelic loss on 18q. This study has identified recurrent clonal DNA abnormalities, suggesting the existence and locations of genes important in uremic hyperparathyroidism. These findings indicate that different molecular pathogenetic processes exist for clonal outgrowth in severe uremic hyperparathyroidism versus sporadic parathyroid adenomas [96].

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. [81, 96, 100]

Recurrent clonal abnormalities have been identified in parathyroid glands of secondary hyperparathyroidism.

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.

Numerous genetic abnormalities are involved in parathyroid tumorigenesis. Various chromosomal regions, various loci containing oncogenes, tumor suppressor genes, and genes for 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, specifically, the monoclonal proliferation, and other non-clonal 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 because of 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.

8.3 Molecular Genetics of Hypoparathyroidism

8.3.1 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 8.4).

8.3.2 Conditions Associated with Hypoparathyroidism

8.3.2.1 Calcium-Sensing Receptor Mutations

The calcium-sensing receptor 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 [154–157].

Occasionally, patients with apparently sporadic idiopathic hypoparathyroidism have an activating mutation of the calcium-sensing receptor. 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, 154, 155, 158].

The most common form of genetic hypoparathyroidism is autosomal dominant hypocalcemia. Autosomal dominant hypocalcemia, because of gain-of-function mutations in the CaR gene, may be asymptomatic or present with neonatal or childhood seizures.

Autosomal dominant hypoparathyroidism associated with short stature and premature osteoarthritis has been reported in a family [159]. 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 autosomal dominant hypocalcemia is associated with an activating mutation in the calcium-sensing receptor; 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 because of increased activation of the calcium-sensing receptor 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 calcium-sensing receptor gene. The therapy in symptomatic patients is to maintain a serum calcium concentration just sufficient enough to improve symptoms.

Acquired defects in the calcium-sensing receptor have been reported in-patients with hypoparathyroidism or hyperparathyroidism, as expression of the calcium-sensing receptor protein may be reduced in adenomas or in chronic renal failure [159].

8.3.2.2 Autoimmune Disorders

Autoimmune Polyglandular Syndrome: Formerly named idiopathic hypoparathyroidism, this disorder represents several syndromes, both acquired and congenital. Autoimmune hypoparathyroidism is a common feature of polyglandular autoimmune syndrome type I and is the most common cause of idiopathic hypoparathyroidism. This is a familial disorder.

Acquired hypoparathyroidism has been thought to have resulted from an autoimmune process, but the self-antigens have not yet been identified.

Two major types of autoimmune polyglandular syndrome are recognized. Hypoparathyroidism is associated primarily with type I, which includes adrenal insufficiency, Grave's disease, muco-cutaneous candidiasis, chronic active hepatitis, hypophysitis, and insulin-dependent-diabetes mellitus among others.

Acquired hypoparathyroidism (AH) has been considered to result from an autoimmune process. From 25 patients with acquired hypoparathyroidism, 17 had type I autoimmune polyglandular syndrome and eight were associated with autoimmune hypoparathyroidism [45]. Five of 25 patients with autoimmune hypoparathyroidism had antibodies against CaR in human parathyroid gland extracts. Fifty six percent of the sera from patients with acquired hypothyroidism 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. This study identified the CaR as an autoantigen in acquired hypoparathyroidism [45].

Parathyroiditis: Autoimmune parathyroiditis is usually associated with hypoparathyroidism, but is occasionally 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.

8.3.2.3 Genetic Disorders of the Parathyroid Hormone and Parathyroid Hormone Receptor

Within this heterogeneous group of diseases, some genetic causes of hypoparathyroidism, such as abnormalities of the PTH gene, have been described [160–162]. 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 parathyroid hormone receptor (PTHr) are identified. Both disorders have opposite effects, and are caused by mutations of the type 1 parathyroid hormone receptor.

Blomstrand' s chondrodystrophy is caused by inactivating mutations of the type 1 parathyroid hormone receptor, and is inherited as an autosomal recessive trait. Jansen's chondrodysplasia is caused by activating mutation of the parathyroid receptor, and is inherited by an autosomal dominant trait [163].

8.3.2.4 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 alpha subunit mediates cyclic AMP stimulation by PTH. Mutations of the GNAS1 gene, which is located on chromosome 20q13.11 and encodes the alpha-subunit of the stimulatory GTP-binding protein, have been identified in the two types of pseudohypoparathyroidism described [164].

Children with pseudohypoparathyroidism present with hypocalcemia and hyperphosphatemia, but PTH levels are elevated, indicating resistance to all the actions of PTH.

Type 1: Pseudohypoparathyroidism type 1 is characterized by a diminished cyclic AMP response to PTH. Two types have been described (types 1a and 1b) as well as pseudopseudohypoparathyroidism. The gene encoding the stimulatory Gs-alpha-one 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 [165]. It is inherited as an autosomal dominant trait. Patients with this form have round faces, short stature, and short metacarpal and metatarsal bones, known as Albright's hereditary osteodystrophy. The hyperphosphatemia induced by this 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 GNAS1 gene, and 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 [164, 166, 167]. It is associated with a defective methylation within GNAS1.

Type 2: Pseudohypoparathyroidism type 2 is characterized by a blunted phosphaturic response to PTH. The pathogenesis is resistant to the intracellular effects of cyclic AMP.

The pseudohypoparathyroid diseases appear to represent a heterogeneous group of disorders with GNAS1 mutations.

8.3.2.5 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 like 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 PTH gene is considered normal [168].

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) [169, 170].

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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Chapter 9
Pathology of Thyroid Gland

Ashraf Khan and Vânia Nosé

9.1 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 fourth and the fifth branchial pouches as the ultimobranchial bodies. The medial analage appears by day 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 weeks; it then grows laterally, and becomes bilobed [1]. Aberrations in the descent of thyroid and pathologic lesions including tumors can arise from this ectopic thyroid tissue. Early during the fifth week, the thyroglossal duct loses its lumen and shortly afterward 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. The causes of the fusion of the lateral and medial analage are unknown. 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 (SCN). 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, 5, 6]. Some have suggested that mixed C-cell and follicular tumors may arise from ultimobranchial body related follicles found associated within SCN [7]. Some of the cells in the SCN on immunohistochemistry stain positive for p63 and may represent the pluripotent stem cells from which tumors may arise [8]. 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 eighth week and by 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 weeks), colloid stage (13–14 weeks), and follicular stage (after 14 weeks) [9]. Evidence of thyroxin comes with the appearance of colloid, and T4 and TSH are detectable in the circulation of human fetuses after 12 weeks [1, 2].

9.2 Normal Anatomy and Histology

The thyroid is normally located in the mid portion 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 adult, the two lobes measure about 2–2.5 cm in width and 4–5 cm in length and in some glands a pyramidal lobe, derived from the distal portion of the thyroglossal duct, extends upward from the isthmus. At birth, the thyroid weighs 1–2 g and it increases to 10–15 g at puberty [10]. In the adult, the normal weight ranges from 15 to 35 g. 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 [11] to about 10–20 g in North American population [12, 13].

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The mean weight is always higher in women and the weight varies with the menstrual cycle [14]. In the elderly, the weight may sometimes reduce to as little as 10 g [1].

On sectioning, 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. The thyroid gland contains three major types of epithelial cells. These include follicular cells, which line the follicles and *secrete* thyroxin and triiodothyronine, C cells which *secrete* calcitonin, and the 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 [15]. The colloid includes thyroglobulin which is a glycoprotein giving it a PAS-positive diastase resistant staining characteristic. The amount of the colloid and the height of the follicular lining cells vary with the functional status of the gland. In a hyperactive state, the follicles are lined by tall cells and have less amount of colloid. In addition to colloid, the follicles contain birefringent calcium oxalate monohydrate crystals, the numbers of which may increase with age. This finding may be useful sometimes to differentiate between thyroid follicles and parathyroid tissue at frozen section [16]. 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]. Because of the intimate development of thyroid with the mesodermal structures of the neck, fat, cartilage, and/or muscle may be found within the thyroid capsule [9]. 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 thyroglobulin, and may also coexpress vimentin. 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 Gremilius 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. In addition, C cells may also stain positive for bombesin, somatostatin, gastrin-releasing peptide, low molecular weight cytokeratin, and CEA on immunohistochemistry. On electron microscopy, the C cells have the characteristic neurosec*ret*ory 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 immuno electronmicroscopy [17]. On in situ hybridization, both calcitonin and CGRP mRNA can be localized in C cells [4].

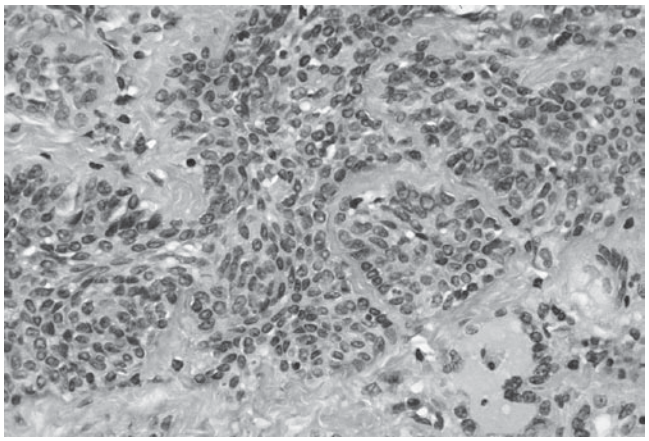

Fig. 9.1 Solid cell nests in a thyroid gland (Hematoxylin & Eosin stain)

The SCN can be seen in up to 60% of thyroid glands in the mid portions of the lateral lobes [18]. 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. 9.1); nuclear grooves may be seen. SCN may sometimes show cystic change and show positive staining reaction with acid mucins. On immunohistochemistry, the SCN are positive for low molecular weight cytokeratin and CEA, focally stain with p63, and show variable staining with calcitonin [1, 4, 8, 19, 20]. The latter finding gives support to the theory of the SCN being derivatives of the ultimobranchial body. On electron microscopy, SCN demonstrate desmosomes, intermediate filaments, and intracytoplasmic microvacuolar structures [21]. In addition, there are dendritic antigen presenting cells that present in the parafollicular thyroid stroma [22, 23]. Mast cells and T lymphocytes may also be seen around the follicles.

9.3 Thyroid Physiology

The thyroid gland produces three hormones. Thyroxine (T4) and tri-iodothyronine (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 thyroid stimulating hormone (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 will be discussed later, may lead to hypothyroidism and goiter. Further, deficiencies in the activity of enzymes involved in thyroid hormone synthesis also lead to hypothyroidism causing increase in TSH which in turn produces hyperplasia and enlargement of the thyroid gland referred to as dyshormonogenetic goiter. Calcitonin plays a role in calcium homeostasis and causes decrease in serum calcium levels.

9.4 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 9.1.

9.4.1 Developmental Abnormalities of the Thyroid

9.4.1.1 Abnormalities of Anatomical Development

Thyroid Agenesis and Hypoplasia

Thyroid aplasia and hypoplasia are the cause of non-goiterous c*ret*inism at birth. In some cases, there may be aplasia of one lobe referred to as hemiagenesis, which is usually seen in left lobe and is associated with normal thyroid function. The prevalence of thyroid hemiagenesis in asymptomatic children in some iodine sufficient population of Europe has been reported to be 0.05% [24]. In patients with di George syndrome, which is associated with third and fourth branchial pouch defect leading to developmental arrest of parathyroid and thymus, there may be arrest in the C cell development [1, 4].

Ectopic Thyroid Tissue

Ectopic thyroid gland (ETG) may be seen anywhere in the midline along the path of the thyroglossal duct, and it may be involved in various thyroid disorders [25]. In such situations, ETG 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 ETG include lingual, sublingual, suprahyoid, and mediastinum. The embryology, clinical presentation, and management of ETG have been recently reviewed by Sood and Kumar [26]. In addition to the midline location, ETG has been reported in submandibular region [27], and other locations including pericardium [4], heart,

Table 9.1 Presentation of Thyroid 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
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, para-aminosalicylic acid, iodides, amiodarone, others)
Secondary hypothyroidism
Pituitary destruction (tumor, infarcts, trauma)
Isolated TSH deficiency
Tertiary hypothyroidism
Hypothalamic destruction (reduced TRH delivery) due to tumor, granuloma and trauma
Euthyroid "Sick" Syndrome: found in severely ill 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
Malignant tumors

porta hepatis [28–30], gallbladder [31], inguinal region [1], vagina [32], sella turcica [33], trachea, and larynx. The tracheal and laryngeal ectopic tissue, if sufficiently large, may produce respiratory symptoms [34, 35]. While cutaneous and subcutaneous thyroid follicular tissue outside the anterior midline neck raises the possibility of metastatic carcinoma, there are recent reports of ectopic thyroid tissue in axilla and posterior neck in which total thyroidectomy failed to reveal an occult tumor, and there was no uptake of radioactive iodine on scan outside the region of the thyroid [36, 37]. Maino et al. suggested that a somatic mutation in a transcription factor important in thyroid migration may play a role in this thyroid heterotopia [37].

Lingual thyroid, resulting from complete arrest of the descent of the medial thyroid analage, is rare with a reported incidence of 1 in 4,500 to 1 in 100,000. In most of the cases, this is the only thyroid gland. Lingual thyroid lacks a capsule, and on histology thyroid follicles are seen mixed with the striated muscle fibers of the tongue. While tumors have also been reported to occur in the lingual thyroid, pathologists must be careful in misinterp*ret*ing thyroid follicles intermingled with muscle as carcinoma. Diagnosis of carcinoma must only be made 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] and may sometimes mimic submandibular gland swelling [27, 38]. 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 peri-thyroidal 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.

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 sub-capsular 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 similar structure in nodes lateral to the jugular vein should be regarded as metastatic carcinoma [1, 3, 4, 39–41]. However, the latter deposits may be seen in cases where multiple serial sections of the thyroid gland failed to reveal any tumor [39, 42, 43]. It is possible that small microscopic thyroid carcinoma in these cases may have involuted leaving a scar behind [39]. It has been suggested that normal thyroid tissue may be transported to the lymph nodes by way of lymphatics [41]. Cacceta et al. reported a case of lateral aberrant thyroid presenting as a mass in the left submandibular area with no evidence of thyroid malignancy; they suggested an anomaly of thyroid embryogenesis primarily in their case involving the left ultimobranchial body [44]. Architecture of the thyroid deposits has been suggested by some to be helpful in differentiating benign thyroid tissue from metastatic deposits, but it has not been reproduced in some other studies [39]. 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 distinguish benign thyroid resting 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 [45].

Thyroglossal Duct Cyst

The thyroglossal duct cyst (TDC) arises from the cystic dilatation of a persistent thyroglossal duct. It is located in the anterior midline of the neck and while they are more common in children, they can present at any age and either sex. On gross examination, the TDC ranges 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 approximately 60% cases. Recurrent infection may cause denudation of the lining of 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. Primary papillary thyroid carcinoma arising from the thyroid tissue within the TDC is a well-recognized complication [46–54]. Other primary thyroid tumors that have been reported to arise in TDC include Hurthle cell adenoma [55] and anaplastic carcinoma [56]. While primary thyroid tumors can occur in the TDC, the possibility of metastasis 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) presence of normal tissue adjacent to the tumor, and (3) failure of careful histopathologic evaluation of the thyroid to reveal a primary carcinoma [2].

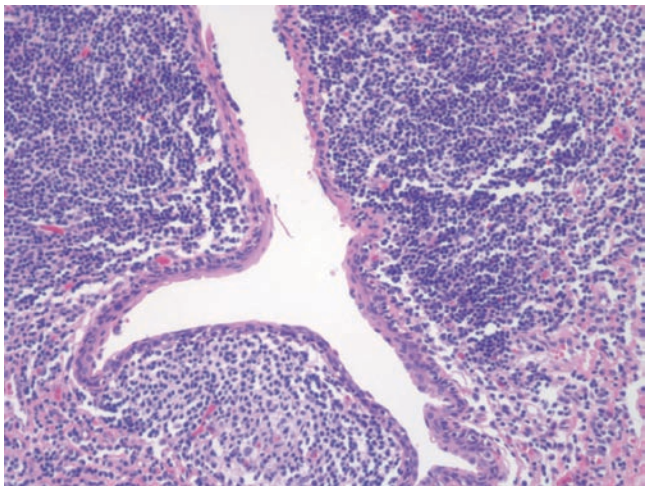

Fig. 9.2 Thyroid gland with a lymphoepithelial cyst (branchial pouch remnant)

Other developmental anomalies of the thyroid gland, which may be encountered in surgical pathology practice, include the finding of a lymphoepithelial cyst similar in morphology to the branchial cleft cyst (Fig. 9.2). These are derived from the branchial pouches four and five that give rise to the ultimobranchial body that is involved in the development of the thyroid gland [57]. Evidence of thyroid development from the fourth branchial pouch is further supported by the finding of ectopic thyroid tissue in branchial cysts in the neck [58].

9.4.1.2 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 (c*ret*inism) and goiter at birth, while partial defects of goiter may develop later in life. Some individuals may be euthyroid with elevated TSH [1]. Pendred's syndrome (PDS), which is characterized by goiter, congenital deafness, and positive perchlorate test, is the most common form of dyshormonogenetic goiter [59]. The underlying molecular abnormality in PDS is mutation in the chloride/iodide transporter pendrin which is encoded by the PDS gene [60–64]. 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 [60, 63]. The abnormality in thyroid hormone synthesis in PDS is thought to be defective thyroid iodine organification which is consistent with a positive perchlorate test [65–67] and is further supported by the finding of impaired thyroid organification in vitro by thyroid tissue taken from patients with PDS [66].

The histo-morphology of the thyroid gland in dyshormonogenetic goiter may sometimes be challenging and an over diagnosis 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, 39, 59, 67]. The diagnosis may sometimes be suggested by FNA in the appropriate clinical setting [68]. True malignant tumors occur in dyshormonogenetic goiters but are extremely rare. Papillary and follicular carcinomas have both been reported in dyshormonogenetic goiter; however, most commonly these tumors are follicular carcinomas, many of which may exhibit an aggressive phenotype including anaplastic transformation [60] A diagnosis of malignancy in such a situation should only be made if there is unequivocal vascular invasion or evidence of distant metastasis [67, 69].

9.4.2 Non-neoplastic Thyroid Disorders

9.4.2.1 Graves' Disease

Definition

Grave's disease is a form of thyroid autoimmune disease characterized by diffuse hyperplasia of the thyroid gland, thyrotoxicosis due to excessive thyroid hormone synthesis, and presence of thyroid associated autoantibodies in the serum.

Historical Background

As early as fifth century B.C. ancient Greeks have been known to describe the combination of goiter, exophthalmos, and palpitations, which is what now recognized as a constellation of symptoms seen in patients with Graves' disease [39]. Parry, in 1786 was the first to recognize and describe a group of patients with a combination of symptoms including rapid heartbeat, goiter, and sometimes exophthalmos [3]. These findings were, however, published in 1825 after Parry's

death by his son in an obscure book [70]. Later Robert Graves in 1835 and Carl Basedow in 1840 also described a group of patients with similar symptoms as described by Parry. As the continental Europeans were not aware of Graves' description, in continental Europe this diseases became known as "Basedow's disease" and some in Europe still use this term rather than "Graves' disease". Both Parry and Graves thought that these symptoms are a result of a cardiac disease and even after Basedow's report, the goiter was not considered of much importance. 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 nineteenth century. By the late nineteenth century when the surgeons were able to remove goiters, it was observed that 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 leads to similar nervousness and weight loss, gave way to the thyroid origin of what is now universally known as Graves' disease [70].

Clinical Features

Graves' disease is seen mostly in women with a female to male ratio of 8:1 and most common age of presentation is in the third or the fourth decade. 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 waisting, weight loss, diffuse goiter, tremors, and eye changes. The clinical manifestations and management of Graves' disease have been recently reviewed and discussed by Brent, GA [71].

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 [72]. Other secondary autoantigens involved in Graves' disease include thyroid peroxidase (TPO), thyroglobulin, and the sodium/iodide co transporter [72, 73]. Two types of TSH receptor antibodies have been identified; these are the TSH receptor-stimulating antibody and the TSH receptor-blocking antibody [72]. Both 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 [74]. TSHR gene single nucleotide polymorphisim (SNP) is associated with the risk of developing Graves' disease [75]. The TSH receptor-stimulating antibody is specific to Graves' disease, while other autoantibodies such as the anti-thyroglobulin, anti-peroxidase antibodies may be seen in addition to Graves' disease in patients with autoimmune thyroiditis and sometimes in normal individuals [72]. The TSH receptor-stimulating antibody functions like TSH causing activation of the adenyl cyclase-cAMP and protein kinase C-phosphoinositide signal transduction pathway leading to increased synthesis and release of thyroid hormone and hyperplasia of thyroid follicular cells [76].

The T cells, predominantly T helper (CD4) cells of both Th1 and Th2 subtype constitute majority of the intra-thyroidal lymphocytes in Graves' disease. Other cell types, which are in minority, include T suppressor (CD8) lymphocytes, B-lymphocytes, and plasma cells. Further, it has been shown that when intra-thyroidal lymphocytes from Graves' disease are grown in vitro, the T cells are primarily Th2 cells with considerable T helper cell activity [77, 78]. What initiates the autoimmune reaction in Graves' disease is not entirely clear. Possible mechanisms include molecular mimicry or cross reactivity resulting from structural similarity between infectious agents and thyroid proteins such as similarity between Yersinia enterocolitica and the TSH receptor [79]. There is no evidence, however, that either Yersinia or other viral infections cause Graves' disease. Other mechanisms suggested for the initiation of the immune reaction in Graves' disease include direct induction of 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 [80, 81]. Risk factors in the causation of Graves' disease include genetic susceptibility such as HLA-B8, HLA-DR3 in white, HLA-DR5 in Japanese, HLA-DR9 in Chinese, HL-B13 and DR5/8 in Koreans [82], infections, stress, sex steroids, pregnancy and post-partum, cigarette smoking, and iodine [72].

Pathology of Graves' Disease

The gross and microscopic appearance of the thyroid gland in Graves' disease varies with the amount and effectiveness of pre-operative medical treatment. In general, the thyroid gland shows diffuse and symmetrical enlargement with a smooth surface (Fig. 9.3). 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 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

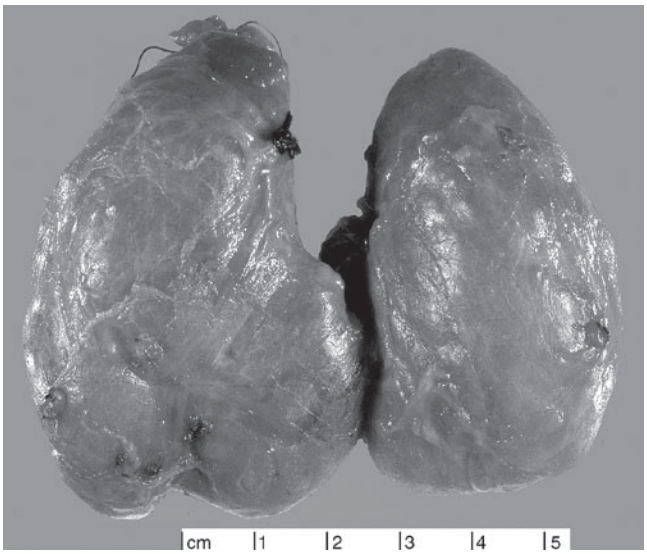

Fig. 9.3 Gross appearance of thyroid gland in Graves' disease

together with the presence of sometimes of optically clear 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, presence of round, basally placed nuclei, and absence of other nuclear changes of papillary carcinoma. Additional histological features in Graves' disease include variable degree of lymphocytic infiltrate in the stroma; the interstitial lymphoid cells are predominantly of T helper (CD4) subtype which aid in the production of antibodies by the B cells [83, 84] Pre-operative medical treatment may alter the characteristic histologic picture in Graves' disease. This may lead to larger follicles, which are filled with colloid, follicular atrophy with Hürthle cell metaplasia mimicking Hashimoto's thyroiditis [85, 86]. Preoperative treatment with radioactive iodine may cause follicular disruption and atrophy with associated Hurthle cell metaplasia, 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 [87]. While the prevalence of palpable thyroid nodule is 3.2–4.7% in iodine sufficient areas [88], in one large multi-institution study the prevalence of palpable thyroid nodules in patients with hyperthyroidism was 15.8% [89]. Other studies have reported similar results [85, 90, 91]. 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% [90]. In a study which included 325 patients with Graves disease, Stocker et al. found papillary thyroid carcinoma in 1.85% of all GD patients, 15.2% of GD patients with cold nodules on scintiscan, 25% GD patients with palpable nodules, and 27.3% of those undergoing surgery [92]. Thus it seems that thyroid scintigraphy is an important preliminary investigation in the evaluation of GD patients and the high prevalence of thyroid cancer in GD patients with cold nodules and palpable nodules warrants further diagnostic evaluation including a fine needle aspiration biopsy (FNAB). In addition to a cold nodule, other significant risk factors for development of cancer in Graves disease include older (>50 years) age [93]. Thyroid cancer arising in the background of GD is thought to be of a more aggressive phenotype compared to the euthyroid patients. The patients may present with nodal and distant metastases, and bilateral and multicentric tumors with invasive growth [87, 94–96]. This however, has not been supported by some other studies [91, 97–99]. 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 as TSH stimulates growth of tumor cells expressing TSH receptor [90]

9.4.2.2 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 9.2. From this table it seems that after infection has been ruled out which anyway is a rare cause of thyroiditis, most other types of thyroiditis have either a definitive autoimmune etiology or are possibly autoimmune

Table 9.2 Classification of thyroiditis

Infectious thyroiditis
Bacterial
Fungal
Mycobacterial
Parasitic
Viral
Autoimmune inflammation
Hashimoto's thyroiditis
Postpartum thyroiditis
Uncertain etiology, possibly autoimmune
Riedel's thyroiditis
Atypical subacute (silent) thyroiditis
Focal lymphocytic thyroiditis
Non immune
Subacute (De Queverain's) thyroiditis

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

Thyroid gland appears to be relatively resistant to infection, making infectious thyroiditis a rare occurrence. Protective mechanisms that have been suggested for making thyroid resistant to infections include rich blood supply to the gland and lymphatic drainage from the gland, high glandular iodine content of the gland which may act as a bactericidal agent, fibrous capsule around the thyroid, and its anatomic separation from other neck structures by fascial planes [100]. The most common predisposing factors to thyroid infections are preexisting thyroid disease including multinodular goiter, Hashimoto's thyroiditis, and thyroid cancer [100–102]. Different modes of infection to thyroid include spread from a primary focus via blood stream or lymphatics, direct spread from adjoining neck structures such as infected TDC, pharynx, or tonsil, and following neck trauma [9, 100]. Direct inoculation following thyroid surgery is extremely rare.

Etiology of Thyroid Infections

Acute bacterial thyroiditis is the most common cause of infectious thyroiditis [84–86]. Most common clinical presentations include thyroid pain, fever, tenderness, and local mechanical compression leading to dysphagia and dysphonia. The pathogens that have been most commonly implicated include *Staphylococcus aureus* and *Streptococcus pyogenes* in adults and alpha and beta-hemolytic *Streptococcus* and a variety of anaerobes in children [100]. Acute thyroiditis may be a result of septic emboli due to Salmonella enteritis in renal transplant patients [103]. Following bacterial infections, fungal infection is the next most common cause of infectious thyroiditis [104]. 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 [100]. Other causes of fungal thyroiditis that have been reported include candida [105], Histoplasma [106], cryptococcus [4], Coccidiodes [107], blastomycosis [108], and Nocardia [109]. Mycobacterial infection of the thyroid is very rare and most often presents in the form of disseminated or miliary tuberculosis [100] or tender enlarged neck mass [110]. Infection with atypical mycobacterium such as *Mycobacterium avium* intracellulare has been reported in patients with AIDS [111] and also in an immunocompetent patient with Hashimoto's thyroiditis [112]. Cases with 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 (CMV) have been reported to cause thyroiditis [4]. Infection with CMV usually occurs in the setting immunosuppressive state. Opportunistic infectious agents that have been isolated from the thyroid in patients with AIDS include CMV [113, 114], *Mycobacterium avium* intracellulare [111], and Pneumocystis [114]. These usually occur in the setting of widely disseminated infections, and CMV and *Pneumocystis 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 [100].

Pathology of Infectious Thyroiditis

Bacterial and fungal infections cause acute suppurative thyroiditis, which on gross examination may show a normal or slightly enlarged gland, which is often associated with focal necrotic areas on sectioning. Histological examination shows acute suppurative inflammation associated with necrotic foci with microabscess. Careful search should be made for microorganisms and special stains performed to look for bacteria and fungi if an infectious etiology is suspected. Fungal infections may sometimes produce a granulomatous inflammation. Mycobacterial infection causes 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 9.3. Infection with CMV will show characterstic intra-cellular inclusions as 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 in patients with AIDS.

Table 9.3 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. Post-operative necrotizing granulomas

Hashimoto's Thyroiditis

Historical Background

In 1912, H. Hashimoto described four cases of goiter for which he coined the term "struma lymphomatosa" (lymphomatous goiter) [115]. All four of Hashimoto's cases were females and showed the histological changes similar to that 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 autoantibodies against thyroglobulin in patient's with Hashimoto's thyroiditis [116]. One year later, Trotter et al. in 1957 identified a second antigen in the microsomal fraction of thyroid homogenates, which proved to be TPO [117].

Clinical Features

Hashimoto's thyroiditis most frequently affects middle-aged females 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 has also identified many cases of thyroiditis with subclinical hypothyroidism characterized with positive anti- TPO and anti-thyroglobulin antibodies in the serum associated with high TSH and normal T4 [118]. 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 [119]. It may also co-exist 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 and up to 5% of first-degree relatives of patients with Hashimoto's thyroiditis may have positive antithyroid antibodies in the serum [119–121]. There is also a high prevalence of autoimmune thyroid disease in patients with Down's syndrome, familial Alzheimer's disease, and Turner's syndrome [122–126].

Pathogenesis of Hashimoto's Thyroiditis

The pathogenesis of Hashimoto's thyroiditis is summarized in Fig. 9.4. Hashimoto's thyroiditis (HT) is a thyroid specific autoimmune disorder in which both humoral and T-cell mediated cellular immune mechanisms play a role. The genes implicated in the pathogenesis of Hashimoto's thyroiditis include HLA-DR locus and non HLA genes such as cytotoxic T lymphocyte antigen (CTLA-4), CD40, protein tyrosine phosphatase-22 (PTPN22), thyroglobulin, and TSHR [127]. The inflammatory process is initiated by the activation of thyroid specific CD4 or T helper cells [128]. The cause of this T helper cell activation is not entirely clear; both viral and bacterial infections have been implicated, but there is no conclusive data to support this [129–131]. There is amino acid sequence homology between thyroid antigen and some infectious agents such as Borrellia and Yersinia species; this crossactivity may trigger the autoimmune inflammation [132]. 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 initiate the formation of autoantibodies against the thyroid autoantigens [133–136]. Once the T helper cells are activated, they stimulate the B cells to produce the antibodies against thyroid antigens such as thyroglobulin, TPO, TSH receptor, and thyroid microsomal antigen leading to antibody mediated thyroid cell injury [137]. Recently, antibodies to Pendrin, apical proteins of thyroid follicular cells responsible of transporting iodine into the cells, have been reported [138]. In addition to this pathway of thyroid cell injury, an alternative pathway involving cytotoxic T (CD8) cell and apoptosis has also been proposed [139–147]. It has been shown that a significant population of intra-thyroidal lymphocytes in HT is of CD8 phenotype having a cytotoxic/suppressor activity [148]. Some studies have shown that follicular cells from tissue samples of HT exhibit strong staining for the death receptor Fas and its ligand FasL; together with a high apoptotic rate as compared with normal controls [149, 150], bcl-2 inhibits apoptosis; immunohistochemical studies have shown a decreased expression of bcl-2 in thyroid follicles from HT patients compared to that in thyroid follicles from normal controls and also from patients with Graves' disease. Further, interfollicular lymphocytes exhibit weak staining for FasL and strong staining for bcl-2 [143, 149, 151]. These data suggest that, in patients with HT, 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 (for review see Wang, SH) [152]. There are also reports of Hashimoto's thyroiditis occurring following subacute thyroiditis [153, 154].

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. The accentuation of the lobulations may make the gland appear nodular on gross examination. Characteristic histologic features of HT include atrophy of the thyroid follicles with oncocytic (Hurthle cell) metaplasia of the follicular epithelium and abundant lymphoplasmacytic infiltrate with lymphoid follicles including germinal centers (Fig. 9.5). In addition there may be varying degrees of fibrosis and foci of squamous metaplasia associated within the atrophic follicles. The peri-thyroidal lymph nodes are generally enlarged

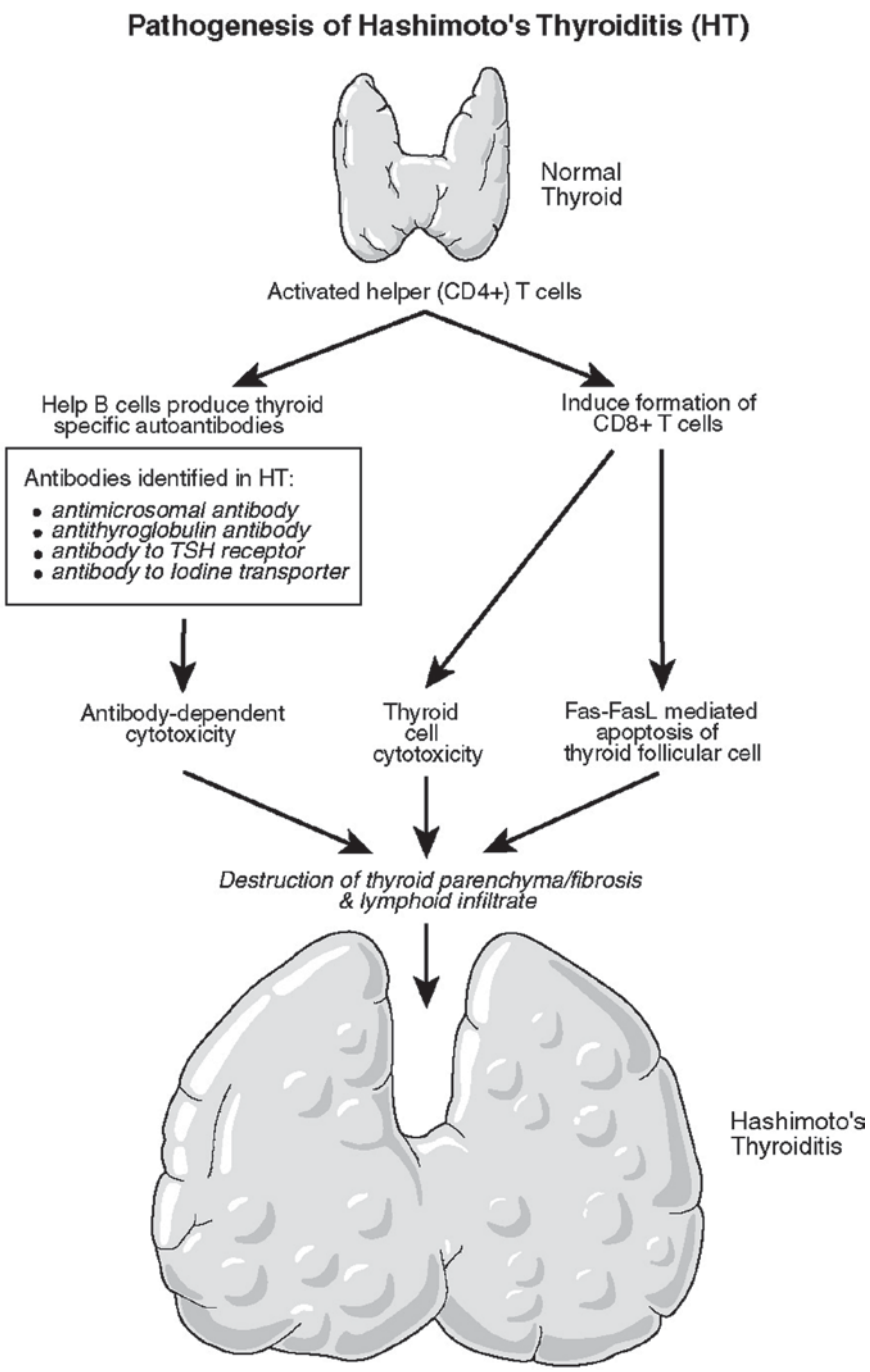

Fig. 9.4 Pathogenesis of Hashimoto's thyroiditis

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. 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 [9, 39, 155, 156]. Therefore, strict histologic criteria should be followed in making the diagnosis of papillary carcinoma in the background of Hashimoto's thyroiditis [39].

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. In addition, there are associated CD8+ cytotoxic/suppressor T cells intermixed with T helper cells, B- cells, and plasma cells [119, 134]. As mentioned earlier, the CD8+ cells are thought to play a role in Fas–FasL

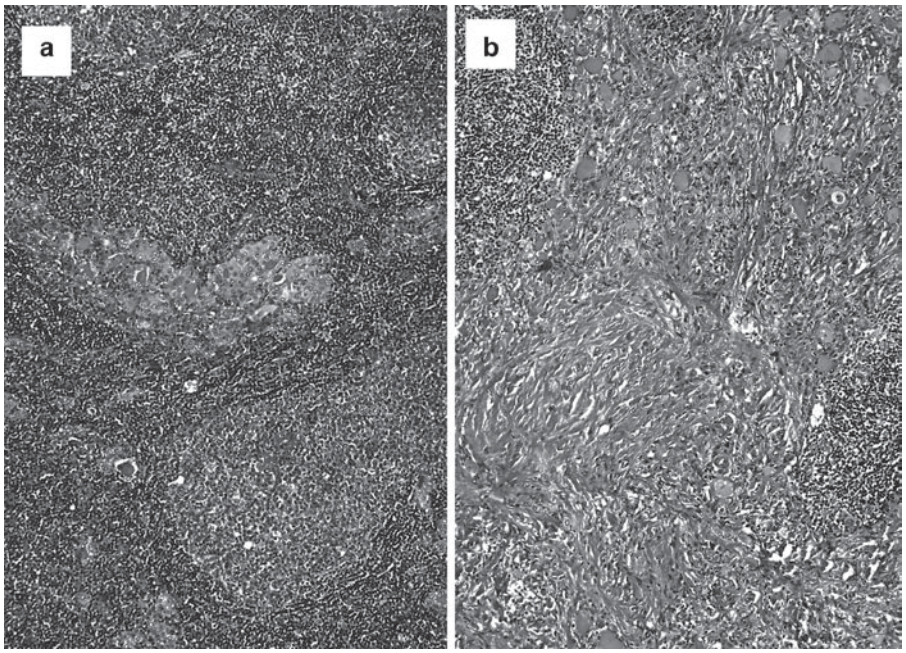

Fig. 9.5 Hashimoto's thyroiditis, (**a**) Classical forma with oncocytic metaplasia and marked lymphoid infiltrate; (**b**) fibrosing variant showing marked fibrosis within the thyroid

mediated thyroid follicular cell apoptosis and cytotoxicity. The plasma cells show reactivity with IgG, IgA, and IgM heavy chains and both kappa and lambda light chains. Immunohistochemistry may also be useful in sometimes differentiating a dense lymphoid infiltrate of HT from lymphoma, which may arise in the background of Hashimoto's thyroiditis. The lymphoid infiltrate in HT is polymorphous as described above and lack evidence of light chain restriction on kappa and lambda 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 HT described above, many variants have been described, which include the following:

Fibrous variant

This comprises approximately 10% cases and is usually seen in the elderly patients who present with markedly enlarged goiter and hypothyroidism [157, 158]. 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. 9.5b), which in some areas has the keloid-like appearance. This fibrotic process is confined to the thyroid capsule, which is an important feature that helps distinguish this form of HT from Riedel's thyroiditis. In Riedel's thyroiditis there is involvement of the neck structures such as muscle, nerves, and sometimes parathyroid by the fibrotic process. Other features that help in this differential diagnosis includes lack of vasculitis and myointimal proliferation, which may be seen in Riedel's thyroiditis. Squamous metaplasia may be seen within the follicles. In addition foci of classic form of HT 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. There is widespread destruction of the thyroid parenchyma with replacement by fibrous stroma. The histological features are similar to the fibrous variant of HT, the only difference being a much smaller gland.

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. The follicular atrophy and oncocytic metaplasia are focal, and hyperplastic changes may be present in the thyroid follicles.

Sub-acute 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" [159]. 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 [159–161]. It has also been seen to be associated with immunosuppressive therapy suggesting an immune mediated etiology [162]. Typically, patients present with thyroid pain, tenderness, high fever, and raised ESR. Subacute thyroiditis has been known to cause thyroid storm [163, 164]. It is a self-limiting disorder in which clinically, three phases of the disease are recognized including hyperthyroid phase, hypothyroid phase, and recovery phase [165, 166]. In the hyperthyroid phase, which is the first one to occur, there is increase in levels of T4 and T3 associated with low radioactive iodine uptake. This thyroid dysfunction 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 [167].

Pathology of Subacute Thyroiditis

The thyroid gland in subacute thyroiditis is asymmetrically enlarged and is firm in consistency. Cut surface shows firm tan gland with variable-sized nodules. 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 comprising 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 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 rightly referred to as *invasive fibrous thyroiditis* [168]. The clinical presenting features include a rapidly enlarging hard neck mass causing compression of the trachea and esophagus, mimicking carcinoma [169]. Hypocalcemia due to hypoparathyroidism may sometimes be present due to the fibrous destruction of parathyroid [170]. There are reports of Riedel's thyroiditis occurring during the course of subacute thyroiditis [171, 172]. The etiology of Riedel's thyroiditis is not known; autoimmune inflammation has been suggested because of the lympho-plasmacytic infiltrate in the thyroid and the presence of thyroid autoantibodies which may be seen in patients with Riedel's thyroiditis [170]. In view of the extensive fibrosis, Riedel's thyroiditis is considered to be part of the idiopathic fibrosing disorders, which include *ret*roperitoneal and mediastinal fibrosis. These latter disorders have been found to be associated with or may develop after the diagnosis of Riedel's thyroiditis [167, 173]. Papi and LiVolsi have recently reviewed and discussed the clinical manifestations, pathogenesis, and management of Riedel's thyroiditis [174].

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 cut surface, the gland is tan-gray in color with loss of normal lobulations. 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 long standing 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 histology alone difficult. In these cases a clinico-pathological correlation is essential to make the diagnosis of Riedel's thyroiditis.

The differential diagnosis of Riedel's thyroiditis includes fibrosing variant of Hashimoto's thyroiditis (see above) and anaplastic carcinoma. While this distinction can be more easily made on thyroid resection specimens, small biopsies may sometimes pose a problem. Riedel's thyroiditis is distinguished from paucicellular variant of anaplastic carcinoma by the absence of nuclear pleomorphism, atypia, and mitoses which are usually seen in the latter.

Other Forms of Thyroiditis

Postpartum Thyroiditis

Postpartum thyroiditis (PPT) 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 [175, 176]. It has been regarded as a variant of Hashimoto's thyroiditis [177]. The autoimmune etiology of PPT 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 PPT is seen associated with thyroid autoimmune disorders such as Graves' disease and primary autoimmune hypothyroidism [176, 178]. PPT occurs in women with positive anti-TPO antibody in early pregnancy, the titers of which decline during pregnancy and then rapidly rise again after delivery [176]. 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. On long term follow-up, persistent hypothyroidism may be present in almost half of PPT patients [179].

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.* 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 [160]. The etiology of silent thyroiditis is uncertain and both autoimmune and viral etiologies have been proposed [180, 181]. 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 either in 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. 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.

Palpation Thyroiditis

Palpation thyroiditis is an incidental finding encountered in surgically resected thyroid specimen. 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 [182]. Granulomas in the thyroid may be seen in other conditions listed in Table 9.3.

9.4.2.3 Goiter

The term goiter refers to enlargement of the thyroid gland; the limit between normal thyroid and goiter is nicely reviewed by Langer [183]. 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 a number of causes such as hereditary deficiency of thyroxin synthesis enzymes (dyshormonogenetic goiter), inflammatory disorders such as Hashimoto's thyroiditis and Graves' disease, thyroid tumors, iodine deficiency, and other goiterogenic agents. In this section the latter group of non-hereditary, non-inflammatory and non-neoplastic lesions will be discussed. In this group the goiter may be simple (non-toxic) or toxic and while the toxic goiters are usually nodular, the simple goiter may be diffuse or multinodular.

Simple (Non-toxic), Diffuse, and Multinodiular Goiter

Simple (non-toxic) 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 nodular thyroid enlargements. 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 more than 10% of children of ages 6–12 within a population have goiter [1]. In both endemic and sporadic goiter multiple factors including relative iodine deficiency, naturally occurring goiterogens, and minimal decrease in 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, it increases with age during childhood and peaks at puberty and child bearing age. The prevalence declines in adults and this decline is more in men than in women. Iodine deficiency in the diet is the most important factor in the development of endemic goiter; this is supported by the finding of decreased prevalence of goiter following iodine

supplementation in the diet. However significant prevalence of goiter persists after iodine prophylaxis suggesting that there may be 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 thioglucosides. The thioglucosides on digestion are converted to thiocyanate and isothiocyanate that is responsible for the anti-thyroid action. Other natural goiterogens include cassava, maize, bamboo shoots, sweet potatoes, and lima beans, which contain the substance cyanoglucosides that has anti-thyroid action. The dietary deficiency of iodine and the consumption of natural goiterogens both play a role in the development of endemic goiter [184]. In a recent study from an endemic goiter region in India, while iodine deficiency may be the primary cause of goiter, the consumption of cyanogenic foods also plays a role in its causation [185].

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 relation to physiological demand for iodine. 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. The cause of sporadic goiter is thought to be relative iodine deficiency. In addition, other factors that play a significant role include presence of dietary goiterogens mentioned above, certain chemicals that interfere with thyroid hormone synthesis – goiterogens, and certain drugs such as paramino-salicylic acid, sulphonylureas, lithium, and excessive iodine. In a large number of patients, no cause can be demonstrated [150]. In the United States population, African-American race, obesity, and increasing age (>40 years) are independent risk factors for goiter [186].

Pathogenesis of Simple Goiter

All non-toxic goiters in the early phase of goiterogenesis are diffuse and with time they increase in size and also become nodular. This may be followed by autonomy in thyroid function independent of TSH sec*reti*on causing sub-clinical thyrotoxicosis and eventually overt thyrotoxicosis.

The various stages of goiterogenesis are shown in Fig. 9.6. In summary, these include (1) growth of the thyroid as a result of stimulatory affect 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 finally (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.

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 with that in sporadic goiter. In early stages, the gland is diffusely enlarged weighing up to 40 g and later in the multinodular goiter stage the weight may range from 60 to 1,000 g [4]. The cut surface in the diffuse goiter is homogenous 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 degree of fibrous tissue and have associated areas of hemorrhage and necrosis (Fig. 9.7). Focal calcification and ossification may sometimes be seen. Some nodules have a thick fibrous capsule and have to be differentiated from follicular neoplasm. 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; this stage is rarely encountered in the 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, and the lining epithelium is flat or low cuboidal. In some large follicles intrafollicular pseudopapillary projections with small lumina may be seen; these are referred to as "Sanderstorm's pollsters" [156]. Thyroid follicular hyperplasia may sometimes mimic neoplastic change and must be distinguished [187]. Secondary degenerative changes in multinodular goiter, which may be encountered, include hemorrhage and necrosis with cholesterol clefts, fibrosis and foci of calcification, and ossification. A dominant nodule with thick fibrous capsule may sometimes be seen which 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

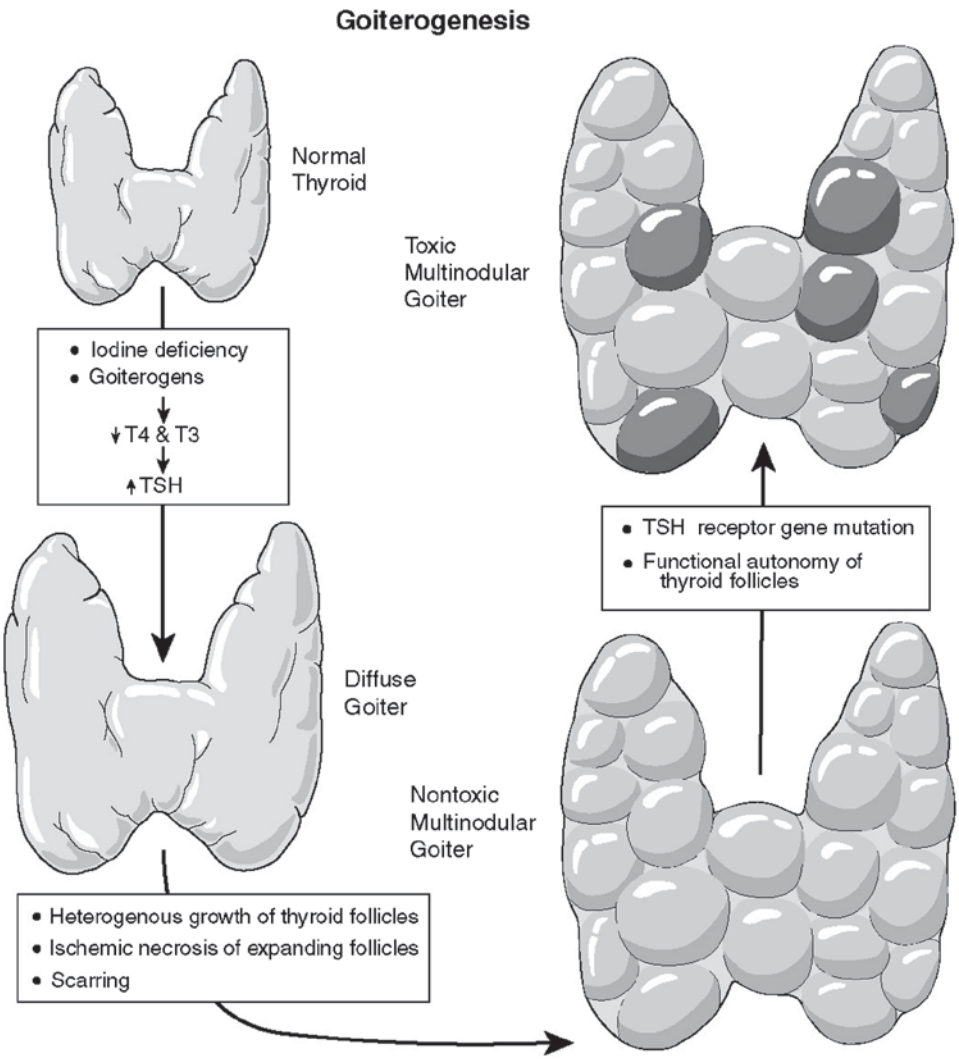

Fig. 9.6 Steps involved in goitrogenesis

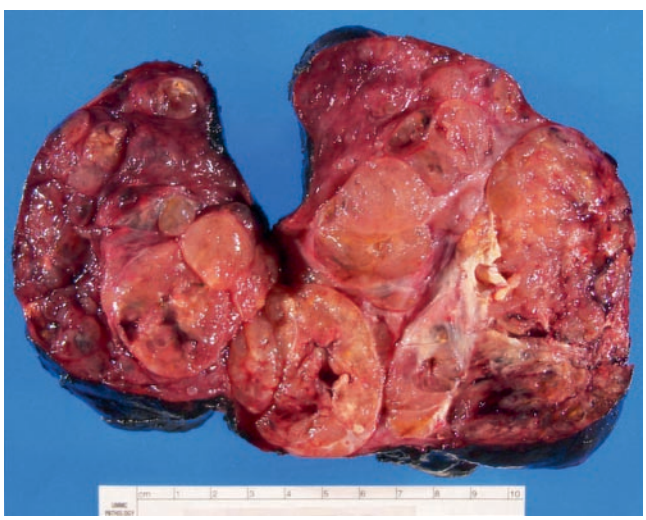

Fig. 9.7 Gross appearance of multinodular goiter showing areas of hemorrhage and necrosis

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 while majority of the hyperplastic nodules are polyclonal both monoclonal and polyclonal populations may coexist within multinodular goiter [188–192]. One study by Chung et al. suggested that 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 [193]. As mentioned above, during goiterogenesis from the diffuse to 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. It is possible that hyperplastic nodule may be the pre-neoplastic 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 [194–198]. The incidence of carcinoma in multinodular goiter is higher in men compared with women and usually occurs in older age group [194–196, 199] Most common malignant tumor arising in multinodular goiter is papillary carcinoma; however, other tumors such as follicular carcinoma, Hurthle cell carcinoma, and medullary carcinoma are also sometimes encountered [198, 200]. In a large prospective cohort study of more than 5,500 cases of thyroid carcinoma treated in the United States during 1996, 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 [201]. The risk of cancer in goiter is increased with higher serum TSH concentration; other independent risk factors include male sex, younger age, and presence of solitary nodules [202]. Furthermore, there seems to be an increase in the incidence of thyroid cancer in goiter, especially microcarcinoma over the past several decades and this may be attributed to improved and increased sampling of surgical resection specimen when rendering a histopathologic diagnosis [203–205]. Multiple foci of follicular pattern lesion with nuclear features of papillary carcinoma may be seen in the background of colloid nodules [206]. In long-standing nodular goiter, the incidence of carcinoma is higher in enlarging solid nodules compared to palpable nodules that decreased in size [207]. Therefore, multinodular goiter with enlarging solid nodules in elderly males carries the highest risk of malignancy, and should be closely followed with FNAB and may be lobectomy if the FNAB is inconclusive. An intra-operative pathology consultation with frozen section and cytology may be helpful, if the FNAB is suggestive of papillary carcinoma, to guide in further intra-operative management [208].

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, with multiple hyperfunctioning areas scattered throughout the gland, barely distinguishable from nonfunctioning nodules [209]. In some cases, toxic adenomas, which 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 (TSHR) have been found in both toxic adenoma and toxic multinodular goiter [209–213]. In addition constitutively activating mutations in the adenylate-cyclase stimulation protein G_s alpha subunit (GNAS) have been seen in toxic adenoma [212, 214]. In a recent study from iodine deficient region of Spain, while TSHR gene mutation was more common, mutations in the GNAS gene were also identified [215]. These TSHR and GNAS mutations in toxic adenoma may also be present irrespective of the iodine supply [216].

Pathological changes in toxic multinodular goiter and non-toxic 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, comprising of follicles with decreased amount of colloid and tall columnar cells lining the follicles. These hyperplastic nodules are present in the background of cold nodules similar to that seen in non-toxic goiter.

9.4.2.4 Other Non-neoplastic Thyroid Disorders

Effect of Drugs on Thyroid

Amiadarone

Amiadarone is an iodine-rich drug comprising 37% iodine by weight that is widely used in the management of cardiac arrhythmia's and congestive heart failure. Because of its rich iodine content, thyroid dysfunction is one of the important side effects of Amiadarone therapy. The effects of Amiadarone on thyroid have been reviewed by Bogazzi et al. and Ursella et al. [217, 218]. These may include a euthyroid state to hypothyroidism and overt thyrotoxicosis; the prevalence of amiadarone induced thyroid dysfunction is higher in elderly patients [219]. Amiadarone induced thyrotoxicosis (AIT) is due to the release of thyroxin as a result of thyroid follicle destruction. 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 that are filled with swollen and foamy cells, which may contain fine pigmented material. These degenerated follicles are associated with areas of fibrosis and nonspecific chronic inflammation (Fig. 9.8) [220].

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 [221].

Pigment in Thyroid

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 previous biopsy site. In these situations iron is seen as coarse brown hemosiderin pigment deposited in macrophages or the stroma. 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; this may on rare occasion cause the gland to appear dark brown on gross examination [9].

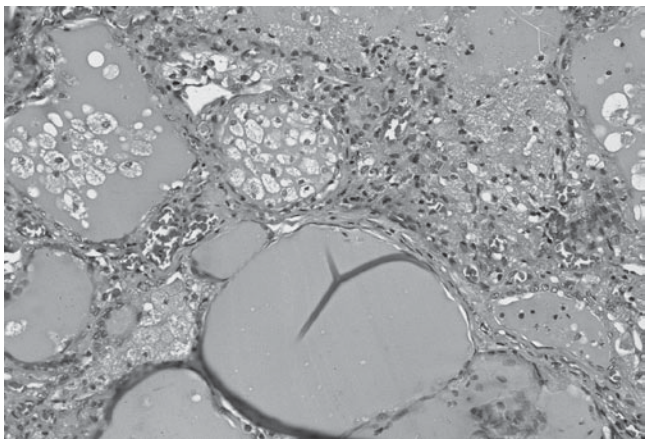

Fig. 9.8 Thyroid gland in Amiodarone associated thyrotoxicosis. Large and degenerating follicles containing foamy cells and evidence of foamy histiocytes and fibrosis in the stroma

Minocycline-Associated Pigment

Minocycline is an antibiotic of tetracycline derivative. Prolonged treatment with this drug causes striking black discoloration of the thyroid referred to as "The Black Thyroid". Histology shows granular black pigment within the apical portion of the thyroid follicular cells [9, 222]. However, tumors arising in the "Black Thyroid" lack the black pigment that is seen only in the residual thyroid (Figs. 9.9a and b) [223].

Lipofuschin

Like 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 thyroid is uncertain, and in most cases no thyroid dysfunction is seen.

9.4.3 Thyroid Tumors

Majority of thyroid tumors are epithelial in origin and can be derived from either the follicular cells or the C (parafollicular) cells. Like in any other organ site, follicular tumors can be either benign or malignant. No benign counterpart of the

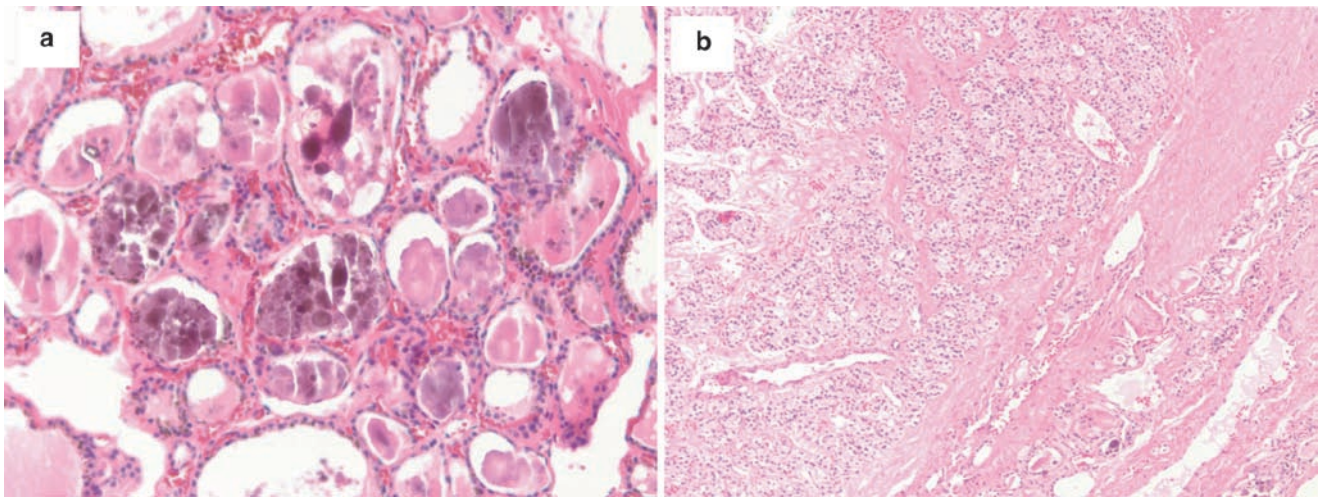

Fig. 9.9 Minocycline induced black thyroid with black pigment within the follicles and follicular epithelium (**a**); same gland with a trabecular adenoma (**b**) which is devoid of the black pigment

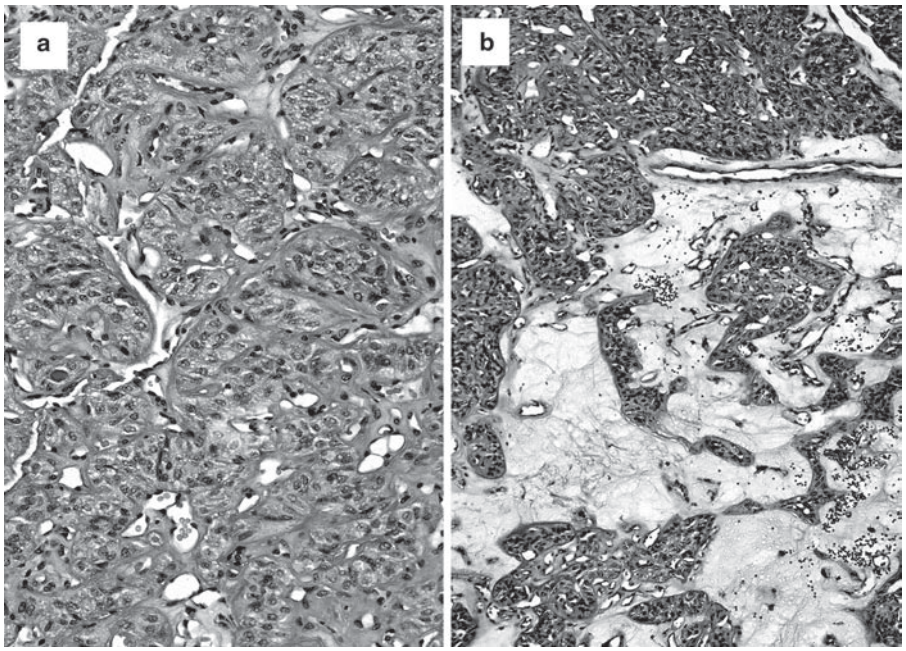

Fig. 9.10 Hyalanizing trabecular tumor: (**a**) peripheral portion of the tumor; (**b**) central portion of the tumor showing stromal hyalinization and edema

C cell tumor is identified; however, various degrees of C cell hyperplasia represent the precancerous stage of medullary carcinoma. A classification of thyroid tumors adapted from the 2004 WHO classification is outlined in Table 9.4. One of the features in the classification of malignant tumors, which seems to be unique to the thyroid, is that it is on the basis of 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, since 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 on the basis of clinical and pathological features, we feel that they should be *ret*ained 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.

Table 9.4 Classification of thyroid tumors

Thyroid adenoma and related tumors
Follicular adenoma
Histologic types
Hyalanizing trabecular tumor
Thyroid carcinomas
Papillary carcinoma
Conventional type
Variants
Follicular carcinoma
Minimally Invasive
Encapsulated angioinvasive
Widely Invasive
Poorly differentiated carcinoma
Insular carcinoma
NOS
Undifferentiated (anaplastic) carcinoma
Squamoid type
Spindle cell (sarcomatoid) type
Small cell type
Giant cell typ
Other Rare Tumors
Squamous cell carcinoma
Mucoepidermoid carcinoma
Sclerosing mucoepidermoid carcinoma with eosinophilia
Mucinous carcinoma
Spindle cell tumor with thymus-like differentiation
Carcinoma showing thymus-like differentiation
Tumors of the C-cells
Medullary carcinoma
Mixed medullary and follicular cell carcinoma
Other thyroid tumors
Mesenchymal tumors
Lymphoma
Metastatic carcinoma

Adapted from WHO Classification (2004)

9.4.3.1 Follicular Adenoma

Clinical Features and Etiology

Follicular adenoma is a benign tumor derived from the follicular cells. It is characterized by an encapsulated tumor showing a follicular architecture with varying degree of cellularity. The incidence of follicular adenoma in autopsy series has ranged from 3 to 4% and no significant geographic variations exist [224, 225]. The dietary iodine has no significant role to play in the causation of conventional follicular adenoma. However, one of the rare variants, toxic adenoma

appears to be more common in iodine deficient areas [226]. Follicular adenoma is more common in females than males and the patients are usually middle aged. While adenomas may occur in children and elderly patients, the chances of an alternative diagnosis of carcinoma are higher in this age group and should be ruled out by further sampling and careful histologic evaluation. The most common clinical presentation of follicular adenoma is a painless nodule in the thyroid, which may sometimes show gradual increase in size and become painful because of hemorrhage and necrosis. The patients are usually euthyroid except in the rare cases of toxic adenoma that present with hyperthyroidism. On radionuclide scan follicular adenoma in most cases presents as a "cold" (hypofunctioning) nodule, though sometimes it may be "cool" or "warm" (functioning) and very rarely "hot" (hyperfunctioning) in the case of toxic adenoma [225].

Pathology of Follicular Adenoma

On gross examination nearly all adenomas are present as a solitary nodule. However, in some instances two or more adenomas may be seen after applying strict criteria for excluding hyperplastic nodules. 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 (FVPC) (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 homogenous cut surface varying in color from grayish white to tan [225]. 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. The solid/trabecular type is also referred to as embryonal type because of its resemblance to thyroid tissue during the prefollicular embryonal stage of intra-uterine life. Similarly microfollicular type is also called fetal type. The follicle size in the normofollicular type is same as that seen in the normal thyroid; therefore, it is also called as simple type and macrofollicular type is called colloid type because of the large follicles mimicking hyperplastic colloid nodule. 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 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 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 [227]. Stromal changes in follicular adenoma include areas of edema, which 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 hemangiopericytoma-like pattern. This close intermingling of follicles with blood vessels may sometimes produce artefactual presence of intravascular epithelial cells, which should not be misdiagnosed as vascular invasion. Other histological changes that may be seen in follicular adenoma include foci of squamous metaplasia, which is exceptionally rare [225], areas of spindle cell metaplasia, which may in some cases involve up to 90% of the lesion [228–230], and papillary architecture mimicking papillary carcinoma [231]. The significance of spindle cell proliferation within a follicular adenoma lies in the importance of distinguishing this metaplastic change from an aggressive malignant process.

Differential Diagnosis of Thyroid Follicular Lesions

Morphology

Thyroid follicular lesions that can sometimes pose a challenge to the surgical pathologist include capsulated or unencapsulated lesions with a follicular architecture comprising varyingly sized follicles filled with variable amount of colloid. The four most important and common lesions that have to be differentiated in this category include *hyperplastic(adenomatous) colloid nodule (HPN)*, *follicular adenoma (FA)*, *follicular carcinoma (FC)* and *follicular variant of papillary carcinoma (FVPC)*. The problems most often encountered are in the distinction of HPN from FA and differentiation of FA from minimally invasive follicular carcinoma (MIFC) and FVPC.

The features that help in the differential diagnosis of these follicular lesions are outlined in Table 9.5. 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 [206]. While the demonstration of capsular and/or vascular invasion is the sole criteria of distinguishing follicular adenoma from follicular carcinoma, what constitutes capsular and vascular

Table 9.5 Differential diagnosis of thyroid follicular lesions

	Diagnosis			
Pathological Features	HCN	FA	FC	FVPC
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 or 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 cells	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-1	Usually negative	Usually negative	Strongly positive	Strongly positive
Galectin 3	Usually negative	Usually negative	Usually positive	Usually positive
CD57	Usually negative	Usually negative	May be positive	Usually positive
CITED 1	Negative	Negative	Usually negative	Positive in subset
P27	Strongly positive	Strongly positive	Focally positive	focally positive
Rb protein	Positive	Positive	Negative	Negative
Cytogenetics				
RET/PTC rearrangement	Absent	Absent	Absent	Present in a subset
BRAF mutation	Absent	Absent	Usually absent	May be present
PAX8/PPARγ rearrangement	Absent	Usually absent	Present in a subset	May be present

HCN hyperplastic colloid nodule; *FA* follicular adenoma; *FC* follicular carcinoma; *FVPC* follicular variant of papillary carcinoma

invasion as discussed later is controversial with significant inter and intra-observer variability and lacks consensus among pathologists, surgeons, and endocrinologists [232–235]. A thick fibrous capsule should raise suspicion and warrants a careful and complete evaluation of the tumor capsule in search for vascular and/or capsular invasion. Multiple cuts of a section may have to be examined. The diagnosis of FVPC is on the basis of the presence of characteristic nuclear features of papillary carcinoma as discussed later [225, 232, 235, 236]. These nuclear changes however may at times be focal and not be well appreciated making diagnosis of FVPC difficult in these cases. In addition to the nuclear changes, the staining quality of the colloid and presence of stromal fibrous bands may sometimes be helpful in the diagnosis of FVPC [225]. While criteria for the diagnosis of MIFC and FVPC have been discussed in multiple studies and clearly laid out as outlined above, from a practical perspective there remains a minor subset of these tumors that constitute the 'gray zone lesions' and are difficult to definitively classify as benign or malignant. Williams et al. initially proposed a terminology for such 'borderline' lesions that was later adopted by the WHO working group and has become a part of their classification. However, this is not accepted by all and still leaves the treating clinicians with the dilemma of how to manage these so-called indeterminate lesions [232, 235]. Immunohistochemistry, which is now widely used in most laboratories and easy to perform, may sometimes help in this differential diagnosis. However, there seems to be some overlap in the immunophenotype and molecular diagnostic tests between morphologically benign, malignant, and so called indeterminate groups of lesions suggesting that if additional larger studies with clinical correlations can reproduce these observations we may in the future be able to reclassify thyroid follicular tumors on the basis of their molecular signature.

Immunohistochemistry

There is a long list of immunohistochemical markers that have been studied to evaluate thyroid follicular lesions and to differentiate benign from malignant lesions; some that have been found to be helpful include galectin-3, HBME-1-1, high molecular weight cytokeratin (CK19), CITED-1, ret/PTC oncogene, peroxisome proliferator-activated receptor γ (PAX8-PPARγ), CD44v6, CD57, and intracellular sodium/iodide symporter (iNIS) (for review see [237–239]). While some have shown great promise especially when used as part of a panel, the 'magic marker' with high sensitivity and 100% specificity still remains elusive. Galectin-3 is a cell adhesion molecule, which is strongly expressed in a subset of follicular carcinoma, and FVPC, while the normal thyroid tissue and benign follicular lesions are either negative or show weak expression [237, 240–250]. As expression of galectin-3 may be seen in benign and normal thyroid tissue, one practical problem seems to be the identification of a semi-quantitative cut-off level of expression as a threshold for diagnosis of malignancy. To overcome this, Liu et al. utilized a receiver operator curve (ROC) analysis and hierarchical cluster analysis to calculate the threshold for protein expression and they concluded that galectin-3 expression when used in a panel with fibronectin-1 and intracellular sodium/iodide symporter had 98% accuracy in diagnosis of thyroid malignancy [246]. In a large multicenter study, the sensitivity and specificity of galectin-3 immunostaining in the diagnosis of thyroid carcinoma were 99% and 98% respectively. In view of this data, Bartolazzi et al. 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 [240]. They referred to these tumors as suspicious adenomas or Potential Early Thyroid Cancers with Molecular Evidence of Transformation (PETCMET). However, these results have not been reproduced in some other studies and galectin-3 expression may be seen in follicular adenoma and hyperplastic nodules; nevertheless a strong and diffuse staining is highly suggestive of carcinoma [237]. In a recent prospective study on FNA samples for selecting patients requiring surgery, the sensitivity of galectin-3 was 78% with 93% specificity, suggesting that while galectin-3 immunostaining cannot replace the morphologic evaluation it can serve as a complimentary test [251]. HBME-1, which is strongly positive in more than 40% of thyroid carcinoma of both follicular and papillary types, is negative or focally positive in hyperplastic colloid nodule and follicular adenoma [237, 252–254]. When used in combination with galectin-3, Rossi et al. found that co-expression of HBME-1 and galectin-3 has a diagnostic accuracy rate of almost 98% for the diagnosis of papillary carcinoma including the follicular variant [255]. Furthermore in follicular tumors of uncertain malignant potential, Papotti et al. found that strong and diffuse expression of galectin-3 and HBME-1 is helpful in classifying some of these lesions as malignant [256]. Cell cycle regulatory proteins such as p27 are down regulated in follicular carcinoma and FVPC and are diffusely positive in hyperplastic nodules and follicular adenoma [257, 258]. Cytokeratin19 and CD57 are also positive in FVPC and are negative or weakly positive in hyperplastic nodule and follicular adenoma [252, 259–263]. However, in another study 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 versus malignant follicular lesion [263]. Also the sensitivity of CK19 expression in papillary carcinoma is around 60% and therefore a negative staining is not helpful [237]. Some authors have suggested that a panel including CK-19, HBME-1, and ret/PTC [252] or CK-19, HBME-1, and CD57 [260] improves sensitivity and specificity in differentiating benign and malignant follicular lesions of the thyroid. Kroll et al. first reported in 2000 that that PAX8-PPARγ fusion oncogene is highly specific for follicular carcinoma [264]. Following Kroll et al.'s report, there have been a number of studies looking at PAX8-PPARγ expression in thyroid follicular tumors and while some have supported their findings, others did not find PAX8-PPARγ fusion to be specific for follicular carcinoma and it may be seen in a significant number of follicular adenoma and FVPC [232, 237, 265–270]. *Ret*inoblastoma protein expression is decreased in FVPC compared to follicular adenoma [271]. Other biomarkers that have been found to be helpful in differentiating benign and malignant thyroid follicular tumors include CITED-1 [272], CD44v6, and CD57 [259, 273–275]. RET/PTC rearrangement and immunohistochemical expression while commonly associated with a subset of conventional papillary thyroid carcinoma [199] have also been reported in FVPC [276].

As no one marker is both highly sensitive and specific, using some in a panel may be more helpful and some studies have found these panels very valuable. These panels include galectin-3 and HBME-1 [256], HBME-1 and galectin-3 [255], galectin-3, fibronectin-1, CITED-1, HBME-1, CK19, and PAX-8-PPARγ [246], galectin-3, fibronectin-1, CITED-1, HBME-1, and CK19 [277–279], galectin-3, CITED-1, HBME-1, CK19, cyclin D1, and p27 [280], and galectin-3 and TPO [281]. We have recently reported another potential marker insulin growth factor 3 mRNA binding protein (IMP3) that has 100% specificity for diagnosing thyroid malignancy with a follicular pattern but has a low sensitivity [282]. We think that careful morphologic evaluation is still most important in the differential diagnosis of follicular thyroid lesions, and immunohistochemistry when used by

employing a panel of markers may be helpful in a small group of lesions where morphology alone is not diagnostic. In our practice, we use a panel of CK19, HBME-1, CITED-1, galectin-3, and IMP3 and have found it to be helpful in the diagnosis of morphologically challenging follicular lesions. However, the search for newer biomarkers is still on and with progress made in the field of molecular diagnostic methods and informatics, gene expression profiling using cDNA microarray is being employed to identify new candidate genes that can be studied at the protein level by immunohistochemistry. Some genes identified by gene expression profiles that have been studied by immunohistochemistry and found useful include PDGF, Bax, P-cadherin and c-MET [247, 283, 284].

9.4.3.2 Follicular Adenoma Variants

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, little intervening stroma, and diffuse cellularity. Pathologists have come to include in this category adenoma showing necrosis and increased mitotic activity [225]. In such cases, careful examination of the capsule should be performed to rule out capsular or vascular invasion. In a recent study, atypical follicular adenoma on immunohistochemistry showed strong and diffuse expression of galectin-3 and HBME-1, similar to the pattern seen in papillary thyroid carcinoma in their series [285]. Furthermore, another study reported that a subset of atypical follicular adenomas showed N2-RAS mutation and RET overexpression similar to the pattern seen in follicular carcinoma and FVPC [286]. These two studies have tried to make a case that atypical follicular adenoma may represent a precursor of differentiated thyroid carcinoma of both follicular and papillary phenotype. In our view these studies highlight the importance of morphologic correlation with immunohistochemistry and other ancillary molecular studies in reaching a diagnosis because relying on immunohistochemistry alone may lead to a false positive diagnosis of thyroid carcinoma. The diagnosis of carcinoma is on the basis of well-established morphological features as described later.

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 sign of malignancy on their own [225].

Adenolipoma and Adenochondroma

Follicular adenomas with interspersed mature fat in between follicles are termed adenolipoma and accordingly tumors with islands of cartilage are called adenochondroma [225, 287].

Toxic Adenoma

Most conventional adenomas are hypofunctioning or "cold" on radionuclide scan. Toxic adenoma is a rare variant comprising approximately 1% of all follicular adenoma, which is "hot" on radionuclide scan, and the patients present with hyperthyroidism. It is also referred to as Plummer's disease. 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. Ultrastructurally, the cells show similar features to that seen in Graves' disease which include increase in rough endoplasmic *reti*culum, well-developed Golgi apparatus, numerous lysosomes, and numerous slender apical microvilli and pseudopods [225]. 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 [209, 210, 212–214]. In a recent study from NW Spain, besides an iodine deficient region in addition to mutation in TSH receptor gene, approximately 5% cases also showed adenylate cyclase-stimulating G alpha protein (GNAS) gene mutation [288].

Signet-Ring Cell Follicular adenoma

This variant of follicular adenoma is characterized by the presence of large intracytoplasmic vacuole causing displacement of the nuclei to one side causing the signet-ring like appearance [289–291]. These foci with signet ring like 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 [39]. 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 [289].

9.4.3.3 Hyalanizing Trabecular Tumor

Hyalanizing trabecular tumor (HTT) of the thyroid is known for the controversy around its terminology mainly because of an apparent uncertainty in the biological behavior of such tumors. Carney et al. in 1987 first coined the term hyalanizing trabecular adenoma (HTA) [292]. However, Carney in a more recent publication recognized that before their report in 1987, similar tumors were reported on three earlier occasions first by Rahel Zipkin in 1905, followed by Pierre Mason in 1922 and in 1982 by Ward and coworkers [293]. Carney et al. in their original paper described a series of 11 encapsulated tumors with a distinctive histology comprising polygonal and fusiform cells arranged in trabeculae separated by thin capillary network and hyalanized amyloid-like stroma which is often calcified (Fig. 9.10a, b). The cells on immunohistochemistry are negative for calcitonin. They considered these tumors as benign and called them adenomas. This pattern is reminiscent of a paraganglioma, and hence the term paraganglioma-like adenoma of the thyroid (PLAT) has been suggested by some [294]. As shown in Fig. 9.11, the cells within these trabecular islands may show longitudinal nuclear grooves, nuclear pseudo-inclusions, psammoma bodies, and paranuclear yellow cytoplasmic bodies [292, 295–297]. 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. These morphological observations were further supported in some studies by the finding of similarities in cytokeratin immunoprofile between papillary carcinoma and HTA, especially cytokeratin 19 that was positive in both [298, 299]. These findings have however not been reproduced in other studies. Hirokawa et al. reported a completely different cytokeratin profile in HTA and papillary carcinoma; in their study, HTAs were all CK-19 negative, while papillary carcinoma was positive [300]. A more recent study reported lack of CK 19 and HBME-1 immunoreactivity in all their HTAs compared to diffuse positive staining noted in all papillary carcinoma [301]. In addition, another 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 [302]. *RET/PTC* rearrangement has been reported in HTA suggesting its similarity to papillary carcinoma at the molecular level [298]. Lloyd reviewed the status of HTA with respect to *RET*/*PTC* rearrangement and emphasized the limitations and practical usefulness of these analyses [303]. In view of the nuclear features resembling papillary thyroid carcinoma together with some studies reporting similar immunophenotype and presence of RET/PTC gene rearrangement in the so called HTA, World Health Organization consensus group in 2004 reclassified these tumors as HTT, which clearly emphasizes the uncertain biologic behavior of these tumors [304]. It is possible that these tumors may represent a group of thyroid tumors with very low malignant potential and an uncertain biology, and further studies utilizing molecular diagnostic techniques with clinical correlation and long follow-up may shed more light in the future [305]. There has been a recent report of hyalanizing trabecular carcinoma presenting as lung metastasis [306]. In this case, however, there was evidence of vascular invasion, with some nuclear groves and rare pseudoinclusions and the tumor was CK19 negative. We think that HTT must be differentiated from medullary carcinoma and poorly differentiated carcinoma and if the latter two are ruled out from being classified as HTT, most of these should have a relatively benign biology. In Carney's experience on the basis of over 120 cases "trabecular tumors of the thyroid that are circumscribed or encapsulated and exhibit intratrabecular hyalinization and PTC-type nuclei are benign neoplasms" [293].

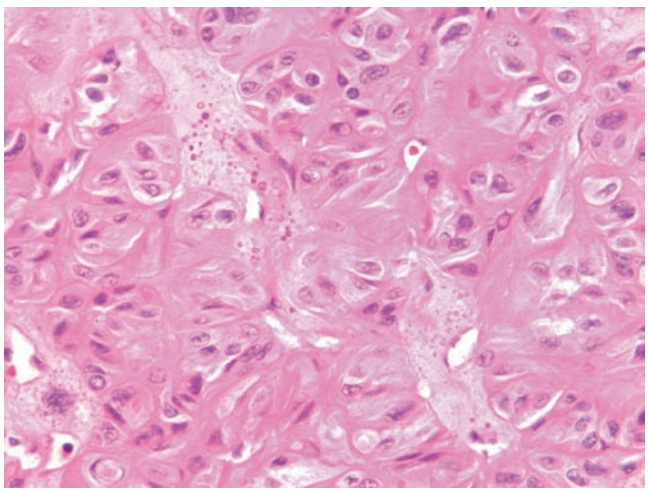

Fig. 9.11 Hyalanizing trabecular tumor with nuclear grooves and nuclear pseudoinclusions

9.4.3.4 Thyroid Carcinoma

Thyroid carcinoma, which constitutes more than 98% of thyroid malignancies, accounts for 1% of all human cancers [201]. At a global level there is significant geographic variation in the incidence of thyroid carcinoma, which ranges from 0.5 to 10 cases per 100,000 [39]. In USA and Europe, the incidence is about 3 per 100,000-population [307]. Also incidence may change when people migrate from one area to another; for example, incidence of thyroid cancer in India is 1.0 but among the south Asians including Indians living in USA the incidence increased to 2.3 per 100,000 [308]. Women are three times more commonly affected than men. While thyroid carcinoma can be seen in all age groups, the

most common age of onset is in the fourth or the fifth decade. 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 seen in the elderly mainly in the seventh and the eighth decades of life [201]. Thyroid carcinoma can arise from either the thyroid hormone producing follicular cells or the calcitonin producing C cells. The later are referred to as medullary carcinoma and may be either 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 including more than 5,500 cases in USA, among the follicular cell derived thyroid carcinomas, the most common is papillary carcinoma (81%), followed by follicular carcinoma (10%), Hurthle cell carcinoma (3.6%), and anaplastic carcinoma (1.7%) [201]. Non-medullary thyroid carcinoma (NMTC) including papillary, follicular, and anaplastic carcinomas may also occur in a familial setting [309–312]. In iodine deficient areas, the incidence of follicular carcinoma is higher and may be as high as 45% [39]; however, in parts of the world with sufficient iodine supplementation the incidence of follicular carcinoma has declined over the past few decades, and one of the reason in addition to iodine supplementation may be increase in the incidence of papillary carcinoma especially its follicular variant [313]. In a recent epidemiological review, Maso et al. concluded that significant risk factors for thyroid carcinoma include exposure to ionizing radiation, and iodine deficiency. Also there seems to be a strong association with history of benign thyroid nodules/adenoma or goiter. Diet rich in vegetables may help prevent thyroid cancer, but a direct causal relationship with diet is yet to be established; worldwide mortality rates for thyroid cancer are 0.8/100,00 for women and 0.4/100,000 in men [314].

Papillary Thyroid Carcinoma

Clinical Features and Etiology

This is the most common form of thyroid carcinoma with no apparently known benign neoplastic counterpart. It most often presents in the fourth and the fifth decades and three times being more common in women than men [201]. Most thyroid tumors in children are papillary carcinomas and rare reports of congenital tumors are also present. Papillary carcinoma may sometimes be seen in a familial setting, and family history of thyroid carcinoma was seen in 4.9% cases of papillary carcinoma in a large cohort of thyroid cancer [39, 201, 312]. In the same cohort, potential risk factors, which were seen associated with papillary carcinoma, include goiter (14.9% cases), thyroiditis (8.1% cases), history of prior exposure to radiation (4.8% cases), and Graves' disease (2.0% cases) [201]. 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 in papillary thyroid carcinogenesis (for review see [315, 316],). 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 [317]. The role of ionizing radiation in the causation of thyroid cancer is well recognized and has been reviewed by Moysich et al. [318]. Furthermore, there is increased incidence of childhood PTC as a second malignancy following radiotherapy involving the head and neck and upper thorax for malignant neoplasm [319].

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. Other 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 often encapsulated and may show partial cystic change [225]. 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 of 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 (Figs. 9.12 and 9.13). These changes include elongation of the nuclei with nuclear groove present along the long axis of the nuclei, optical clearing of the 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. On immunohistochemical studies β-catenin localization has been reported in these nuclear pseudoinclusions suggesting its role in nuclear envelope changes [320, 321]. While nuclear grooves may be seen in other types of thyroid lesions, both neoplastic and non-neoplastic [322], the overall appearance of the lesion both at architectural and cytologic level is helpful in distinguishing these conditions from papillary thyroid carcinoma. These nuclei in papillary carcinoma often show overlapping

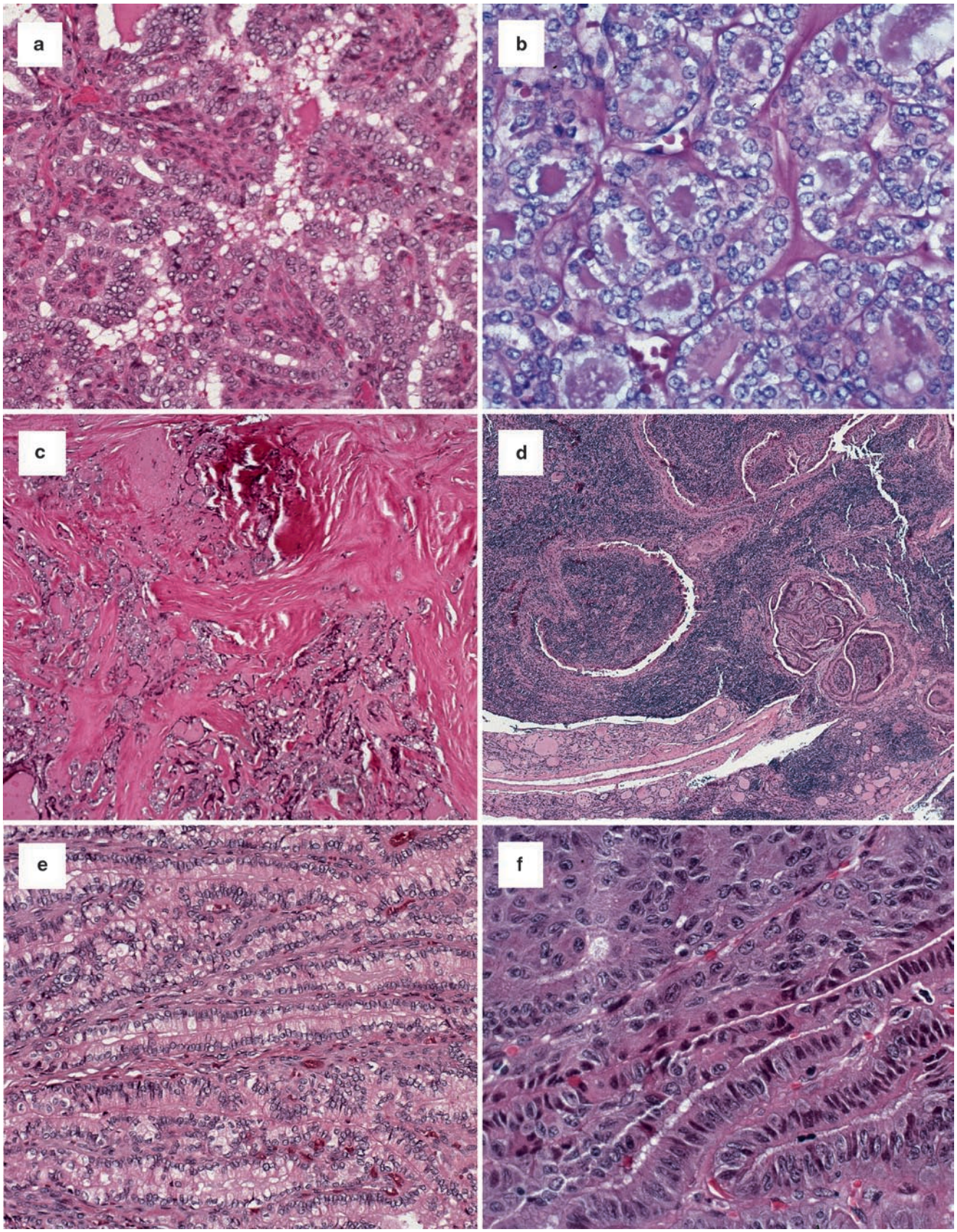

Fig. 9.12 Papillary thyroid carcinoma: (**a**) conventional type; (**b**) follicular variant; (**c**) diffuse sclerosing variant; (**d**) Warthin-like papillary carcinoma; (**e**) Columnar cell variant; (**f**) Tall cell variant

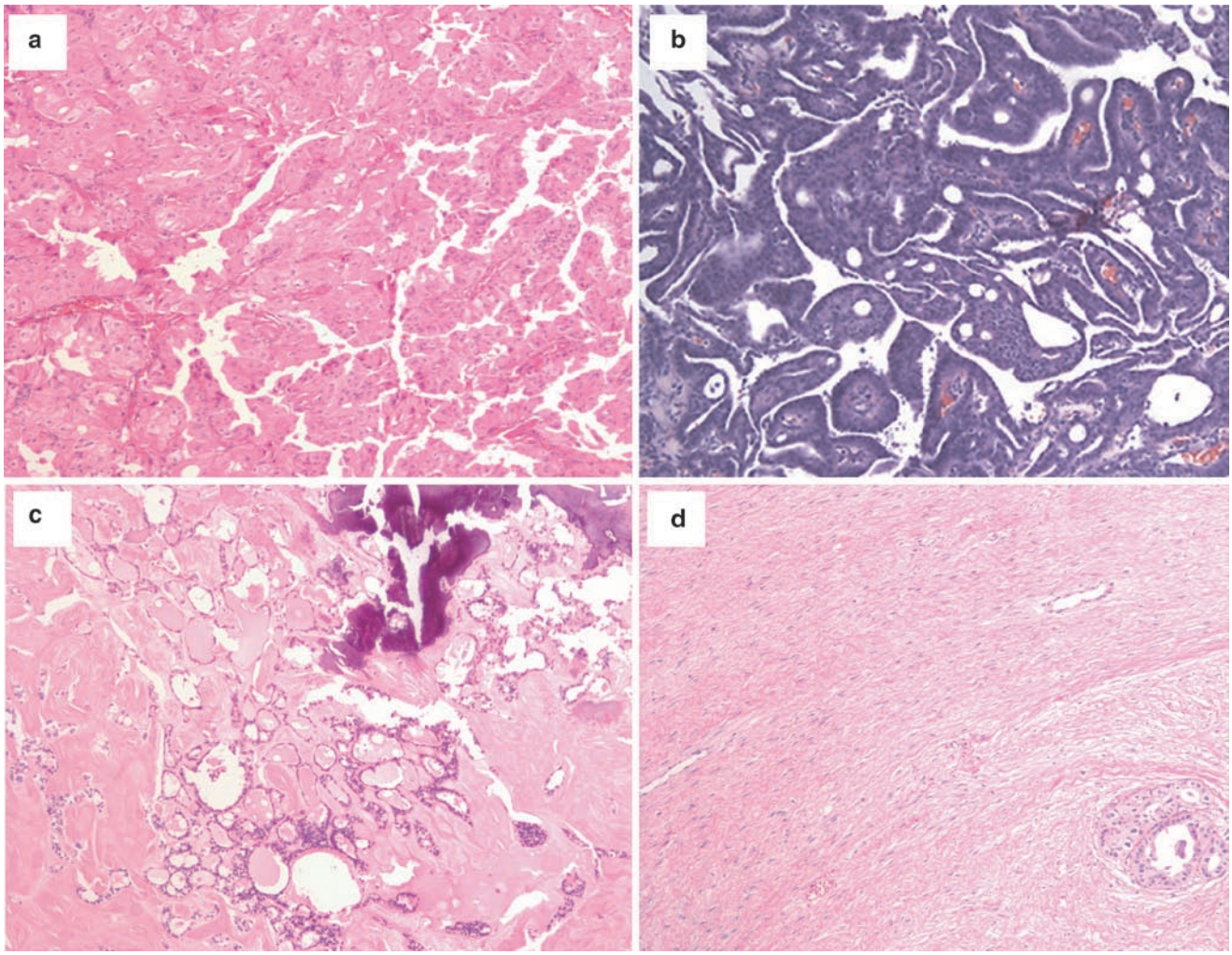

Fig. 9.13 Papillary thyroid carcinoma variants: (**a**) Oncocytic variant; (**b**) Cribriform-morular variant; (**c**) Follicular variant diffuse type with calcification and ossification; (**d**) Papillary carcinoma with nodular fascitis like stroma

features. Additional histologic changes include the presence of psammoma bodies which are concentric calcific bodies within in the stroma of the papillae, 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 [39, 225]. Papillary carcinoma may show multi-centricity and may be present in bilateral lobes justifying the near total thyroidectomy that most surgeons prefer especially for tumors larger than 1.0 cm in size. It is not completely clear whether the multicentricitry is due to intra thyroidal spread of the tumor via lymphatics, or it 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. In a recent study on multifocal PTC, molecular analysis was able to separate intrathyroidal metastasis (ITM) seen in 67% cases by having a similar profile of loss of heterozygosities (LOH) and presence of BRAF mutation from other cases which were labeled as multiple independent primaries; cases with ITM in their study had a higher incidence of lymph node metastasis (LNM) [323]. Papillary carcinoma usually disseminates through lymphatics and it is not uncommon to find metastases in regional lymph nodes. Some authors have suggested sentinel lymph node biopsy as an accurate method for the diagnosis of metastatic papillary carcinoma avoiding significant morbidity associated with lymph node dissection [324–328].

On electron microscopy, the nuclear membrane of papillary carcinoma is highly irregular showing infolding, the chromatin is scant, and nuclear pseudoinclusions containing cytoplasmic contents are seen [39]. Numerous immunohistochemical markers such as cytokeratin 19, HBME-1, CD57, CD44, galectin 3, p27, cyclin D1, Retinoblastoma protein, and CITED have been reported to help in the diagnosis of papillary thyroid carcinoma, especially in the follicular patterned lesions. While some have shown promising results

especially when used in a panel as discussed below, no single marker on its own is absolutely specific and nuclear cytomorphology still may be the gold standard for the diagnosis of papillary thyroid carcinoma. Some recent studies have shown deregulation of a subset of microRNA (miRNA) in PTC, which may potentially be used in FNA samples and aid in preoperative diagnosis [329–331].

Histologic Variants of Papillary Carcinoma

While the characteristic nuclear features described above are common to all histologic types of papillary carcinoma, a number of variants have been described on the basis of either size or architectural patterns of the tumor (Figs. 9.12 and 9.13). The diagnosis of some these variants is important because they behave in a more aggressive fashion compared to the conventional papillary carcinoma and the knowledge of some other variants is important to prevent misdiagnosis.

Papillary microcarcinoma

Papillary thyroid microcarcinoma is defined as tumor less than 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 [39]. Because of its high prevalence up to 35% in autopsy series, and in up to 24% in total thyroidectomies for unrelated benign condition, this tumor is regarded as an indolent tumor with a relatively benign course [332–334]. For the same reason some have suggested that this may be an earlier stage in the papillary carcinogenesis [314]. However, up to 11% of microcarcinoma may show lymph node metastases and local recurrence, which are usually seen in multifocal and bilateral tumors [39]. In one study tumor size ≥0.8 cm was significantly associated with more aggressive disease [335]. However, in a recent meta-analysis of the literature, significant risk factors for recurrence included younger age group, clinically overt tumor, multifocality, and lymph node involvement at diagnosis; tumor size was not associated with recurrence [336].

Follicular variant of papillary carcinoma

This is the most common variant of papillary carcinoma and is also the one that has generated a lot of controversy in its diagnosis. While Crile and Hazard had recognized carcinoma with a follicular pattern as early as 1953, the term FVPC was first proposed by Lindsay in 1960 [337–339], but it was still regarded as a type of follicular carcinoma. The WHO in 1974 recognized the entity as FVPC in its classification of thyroid tumors [340]. Later in 1977 Chem and Rosai described six additional cases elaborating the morphologic features and proposed the use of the terminology FVPC proposed by Lindsay [341]. Since the description by Chem 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 [342]. The follicular pattern in this tumor may be microfollicular, normofollicular, macrofollicular, or a mixed pattern. These tumors may be infiltrative (noncapsulated) or be partially or completely encapsulated . In a recent study from Memorial Sloan-Kettering Cancer Center, FVPC with an infiltrative and noncapsulated pattern showed significantly higher rate of regional LNM (65% vs. 5%), intratumoral fibrosis (88% vs. 18%), exrathyroidal extension (65% vs. 5%), and positive margins (50% vs. 2%) compared to encapsulated tumors [343]. One of the most controversial and challenging diagnoses in thyroid surgical pathology may be the identification focal nuclear changes of papillary carcinoma in an encapsulated follicular pattern lesion which many pathologists may have faced [344]. It has been suggested that there may be a tendency to overdiagnose FVPC [345], 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 and interobserver variability [346, 347]. As 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 [39]. The importance of diagnosing these lesions as encapsulated FVPC cannot be overemphasized because these tumors may later present with bone metastases some doing so 15–17 years after the initial diagnosis justifying their appropriate treatment at the time of presentation [348]. 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) [344]. The features that may help in the differential diagnosis of these follicular pattern lesions are outlined in Table 9.5.

In addition to the encapsulated pattern of FVPC, another distinct pattern that has been described in FVPC includes a diffuse variant which involves the entire thyroid, may be associated with stromal calcification and ossification and shows a high incidence of lymph node, distant metastases (Fig. 9.13c) [349, 350], and the macrofollicular variant described by Albores-Saavedra et al. which should be distinguished from colloid nodule [351, 352]. Some cases of macrofollicular variant in a later publication by the same authors showed focal transformation into a poorly differentiated (insular) phenotype with evidence of lung and bone metastases [353]. While 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. In a recent Japanese study FVPC was associated with a higher incidence of distant metastasis and more aggressive behavior

compared to conventional PTC [354]. Other studies with long-term follow-up have reported similar 10 and 15-year disease specific survival in both FVPC and conventional PTC [355, 356]. Furthermore, immunophenotypically the keratin expression profile of these tumors resembles that of the conventional papillary carcinoma as opposed to the follicular carcinoma [225]. One recent study has also showed overexpression of microRNA (miR-146b) similar to that seen in conventional PTC [329]. However there is also evidence that FVPC shares some features with follicular carcinoma such as higher frequency of systemic spread, presence of PAX8-PPARγ rearrangement seen in approximately one-third of the patients and higher frequency of ras mutations compared to conventional PTC [265, 266, 357, 358]. These morphologic and molecular features suggest that FVPC may be related to both conventional PTC and follicular carcinoma and larger series with clinicopathological correlations and molecular data may shed some more light in the classification of these tumors.

Tall cell variant of papillary carcinoma

Tall cell variant (TCV) is one of the more aggressive variants of papillary carcinoma, first described in 1976 by Hawk and Hazard [359]. In one series of 650 PTC over a 30-year period, TCV comprised 4% of all PTC [360]. These tumors usually present in the elderly and are more common in males. On gross examination, they are usually larger than 5.0 cm in size and on histologic examination show papillary structures lined by elongated tumor cells with height being at least twice that of the width having an eosinophilic cytoplasm and characteristic nuclear features of papillary carcinoma (Fig. 9.12f). Ultrastructurally there are increased mitochondria in the cytoplasm, but it is less than that seen in Hurthle cells [39]. The TCV of papillary carcinoma is associated with adverse prognostic features such as large tumor size, extrathyroiodal extension, vascular invasion and with high incidence of locoregional recurrence, distant metastasis, and shorter disease free survival [361–368]. Furthermore TCV even without extrathyroidal extension has more aggressive phenotype compared to conventional PTC independent of age, gender, and tumor size [369]. In addition evaluation of cell cycle regulatory proteins such as p27, Ki67 cyclin D1, P53, and eukaryotic translation initiation factors 4E and 2 alpha expression, shows a profile which is similar to that seen in thyroid tumors with an unfavorable prognosis [370–374]. The TCV has been found to be associated with squamous cell and Hurthle cell carcinoma of thyroid, which are both more aggressive types of thyroid carcinoma [370, 375].

Columnar cell variant of papillary carcinoma

This is one of the rare and also a more aggressive variant of papillary carcinoma first decried by Evans [39]. LiVolsi later highlighted an important morphologic alteration in these cells, which includes subnuclear vacuolation mimicking early secretory endometrium (Fig. 9.12e) [9]. This latter feature is important and helps in differentiating this from the TCV. Encapsulated tumors tend to have a better prognosis compared to tumors without a capsule that are associated with extrathyroidal extension and higher local regional recurrence [376, 377]. In metastatic sites, however, because of the lack of colloid and presence cytoplasmic vacuolation these tumors may sometimes be confused with other primaries, and to complicate matters further this tumor may show variable thyroglobulin immunostaining but TTF-1 immunoexpression is more consistent [378]. Clinicopathologic correlation and positive TTF1 and thyroglobulin immunostaining are helpful in these cases to make the diagnosis of metastatic thyroid carcinoma.

Cribriform morular variant of papillary carcinoma

This is a rare but distinct variant of papillary carcinoma, which may be associated with familial adenomatous polyposis (FAP) and germ-line mutations in the APC gene [379–384]. Tumors in FAP patients that do not show APC gene mutation may have aberrant nuclear accumulation of mutant β-catenin that is thought to play a role in the histogenesis of these tumors [385]. Sporadic cribriform-morular variants of PC have also been reported without associated FAP, but the diagnosis of this tumor warrants a full workup to rule out associated colonic polyposis [386–389]. Cribriform variant of PC occurs almost exclusively in women and may be solitary or multifocal. They may be encapsulated and are histologically characterized by a cribriform, solid/trabecular, morular (squamoid) growth pattern with intermixed papillary and follicular areas (Fig. 9.13b). The diagnosis of papillary carcinoma is on the basis of the finding of characteristic nuclear features. 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 PC [386].

Oncocytic variant of papillary carcinoma

This variant of papillary carcinoma usually displays papillary architecture in which cubo-columnar cells with eosinophilic cytoplasm and nuclear features of papillary carcinoma line the papillae. While majority have a papillary morphology, areas with follicular pattern or in some cases predominantly a follicular-pattern tumor may be seen (Fig. 9.13a) [390]. This tumor should be differentiated from papillary variant of Hurthle cell carcinoma and TCV of PTC because of different clinical course of the two tumors. In the former characteristic nuclear features of PC are not seen, the papillary structures are not true papillae and there is very little stroma. In TCV of PC, the height of the cells is at least two times the width. Oncocytic variant may sometimes be associated with the TCV of PTC and may show higher propensity for extrathyroidal invasion and vascular invasion compared

to the conventional PTC [391]. Molecular analysis of these tumors has revealed the presence of both RET/PTC rearrangement and BRAF mutations [392, 393]. However it seems tumors with a follicular architecture are less likely to have BRAF mutation compared to tumors with a papillary architecture, again making a case for a different biology of the FVPC [394].

Solid variant of papillary carcinoma

This variant of papillary carcinoma is most often seen in the pediatric population and is the most common variant of PTC in children following the radiation exposure due to the Chernobyl nuclear disaster [395–397]. However, a more recent study found no difference between childhood PTC tumor types in patients who were exposed to the nuclear disaster and those from the same countries who were not, suggesting that there may be other geographic variations such as dietary iodine that may play a role in the genesis of these tumors [398]. The solid variant of PTC is associated with adverse prognostic factors such as lymph node metastases, extrathyroidal extension and venous invasion. On histology they 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 [399]. They are associated with slightly higher incidence of distant metastases and less favorable prognosis than conventional PC [400], 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 [39, 225, 401].

Warthin-like 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 "Warthin-like thyroid tumor" [402]. 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. 9.12d). This tumor behaves like the conventional PC [403–406]. Lam et al. reported a case with progression to anaplastic carcinoma leading to systemic spread and death 18 months after first surgery [360].

Papillary carcinoma with nodular fascitis-like stroma

This is a rare variant of PTC 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 (Fig. 9.13d) [407–412]. In surgical pathology practice it is important to recognize this variant so that in the presence of exuberant fibroblastic proliferation careful search may be made to look for tumor islands. Furthermore, this variant must be distinguished and not misdiagnosed as a more aggressive anaplastic thyroid carcinoma [408]. Another differential diagnosis includes solitary fibrous tumor of the thyroid [413]. The presence of fibroblastic stroma may pose problems in its diagnosis on FNAB [414]. Few case studies available show the behavior of this variant similar to that of the conventional PTC [407].

Diffuse sclerosing variant of papillary carcinoma

This variant of PC 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. 9.12c). Focal squamous metaplasia, numerous psammoma bodies, and lymphocytic infiltrate are additional histologic features. Because of the diffuse infiltrative pattern, these tumors may not produce a clinically palpable mass and so these patients tend to present at a later stage [225]. On ultrasound, they usually manifest diffuse calcification associated with a suspicious mass, which in children and young adults must alert to this diagnosis [415]. The overall survival in these patients is similar to that in the conventional PTC of same stage, but as they tend to present at a higher stage with extrathyroidal extension and higher frequency of LNM a more aggressive surgical treatment is recommended in these patients [416–418]. In Thompson et al.'s series of 22 cases, 5-year disease free survival was seen in 95% and one patient died of disease following transformation of the tumor to squamous cell carcinoma [418].

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-year survival rate is 90–95% and 10-year survival rate is 80–95%. Independent adverse prognostic factors include older age (above 45 years), extrathyroidal spread, aggressive histologic variants, and distant metastases [225, 307]. The significance of LNM as a prognostic factor and the extent of lymph node dissection needed at surgery have been subject of debate. Some studies have shown that while 10-year probability for recurrence is significantly higher in patients with macroscopic LNM, the figures for microscopic LNM were similar to patients with no LNM and so in the absence of gross nodal disease a limited prophylactic node dissection may be sufficient [419–421]. While some studies have shown a higher incidence of distance metastasis in FVPC compared to the conventional PTC [354], prognosis in this variant is not different from the conventional type PTC of the same stage [422].

Follicular Carcinoma

Follicular carcinoma is 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 FVPC gained recognition after its description by Chem and Rosai in 1977 [234, 313, 341]. However, follicular carcinoma is still the second commonest malignant tumor after papillary carcinoma accounting for 10% of all thyroid cancers in the USA [201]. In other parts of the world, especially in areas with iodine deficiency, the incidence of follicular carcinoma is higher and may be up to 45% of all thyroid cancers [39]. It has been shown that addition of iodine to the diet results in relative increase in papillary carcinoma and corresponding decrease in follicular carcinoma [225]. Follicular carcinoma may occur at any age but most commonly presents in the fifth decade; it is rare in childhood unlike PTC and has the same female predilection of 3:1 as PTC [201, 423–425]. It typically presents as a solitary thyroid nodule that is usually "cold" on radionuclide scan; sometimes bone metastases may be the presenting feature and unlike PTC follicular carcinoma are not clinically occult [225].

According to the WHO classification of thyroid tumors, follicular carcinoma is subdivided into two groups namely *minimally invasive or encapsulated* and *widely invasive* follicular carcinoma [426]. The distinction into these two groups is important because of their significantly different clinical behavior and treatment [234, 427]. However, what constitutes MIFC has been the subject of debate and lacks uniform criteria among pathologists, endocrinologists, and surgeons [234, 428] Some authors have suggested that as angioinvasion is associated with a worse outcome these tumors be separated from minimally invasive carcinoma and terms such as encapsulated angioinvasive carcinoma [429, 430] and moderately invasive [431] have been proposed.

Minimally Invasive (Encapsulated) Follicular Carcinoma

MIFC is defined as an encapsulated follicular tumor showing foci of full thickness capsular invasion (Fig. 9.14b) and/or vascular invasion within or outside the capsule. The capsule in most of these tumors is thick, but cases with thin capsule or uneven poorly formed capsule may also be seen [428]. However, what constitutes *capsular invasion* lacks consensus and both partial and full thickness capsule invasions have been cited as criteria for capsular invasion in the past [39]. A survey of endocrine pathologists revealed lack of consensus on the definition of capsular invasion to diagnose MIFC [234]. In the recent WHO series on classification of tumors, capsular invasion is defined as penetration through the capsule unassociated with previous FNAB [304]. There however remains a significant interobserver variability in diagnosis of MIFC [233]. For encapsulated

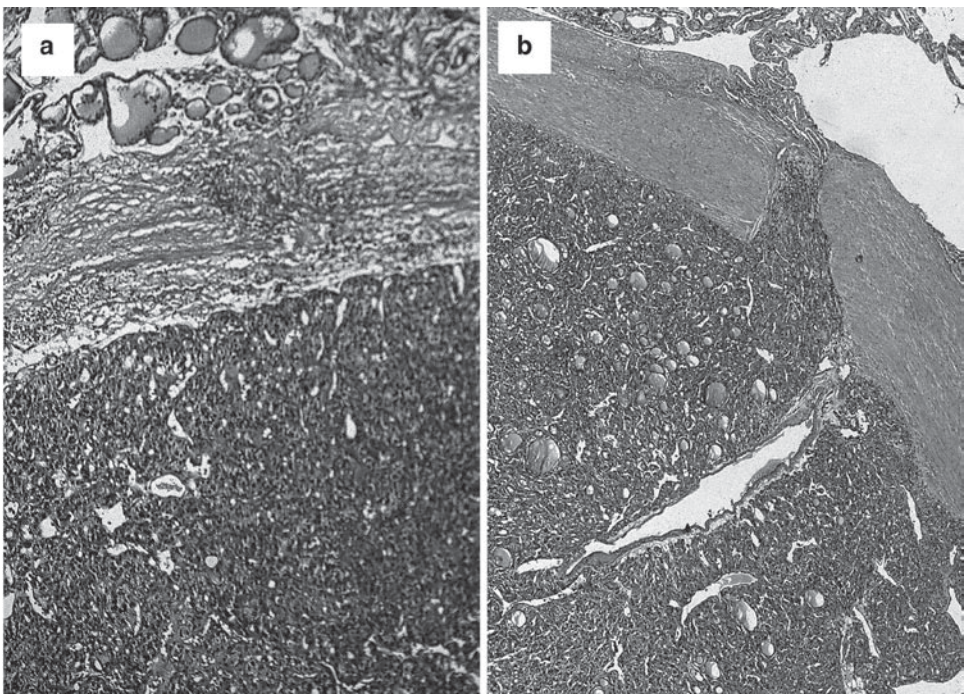

Fig. 9.14 Microfollicular adenoma (**a**); and minimally invasive follicular carcinoma showing a thick fibrous capsule and a focus of transcapsular invasion (**b**)

follicular tumors with questionable capsular invasion showing no vascular invasion some authors have suggested that they should be classified as follicular tumor of uncertain malignant potential (FT-UMP) [344]. There are reports of follicular carcinoma with capsular invasion only showing distant metastases justifying their designation as carcinoma and appropriate management [432, 433]. 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 [225, 304]. In minimally invasive carcinoma, the blood vessels outside the capsule are of small or medium size and lack a continuous muscular layer [427, 428]. The tumor cells should be present within the vascular lumen like a fibrin thrombus and show attachment to the endothelial surface at some point, which helps to avoid overdiagnosis because of artefactual presence of tumor cells in the blood vessel as a result of dislodging of tumor cells during handling of the specimen at surgery or gross examination. At times tumor cells are seen pushing into the 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 [225]. LiVolsi has suggested an approach, which seems practical in that tumors with capsular invasion only are regarded as minimally invasive carcinoma and tumors with vascular invasion are designated as angioinvasive grossly encapsulated follicular carcinomas. The angioinvasive tumors have a capacity for hematogenous spread; 50% of these patients die of tumor in 10 years and patients with capsular invasion only have a better prognosis [39]. Further larger series with long-term outcome data applying this classification may be useful in validating its clinical significance. Prognosis in MIFC is excellent and some have suggested that patients may not need to undergo complete thyroidectomy following a diagnosis of MIFC in a lobectomy [434]. Thomson et al. in a 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 years. Four patients showed recurrent disease and one of whom died after 15 years; this latter patient was a woman who was 49 years old at presentation and had a large 7.0 cm tumor [428]. Distant metastases in MIFC are seen more often in tumors showing vascular invasion, so it may be best to regard this as a distinct group as suggested earlier when evaluating the prognosis [435]. In view of the focal nature of capsular and vascular invasion it is important that encapsulated follicular tumors should be evaluated by thorough sampling, which must include examination of the entire capsule. 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 as discussed later includes other follicular patterned lesions such as follicular adenoma, FVPC, and hyperplastic colloid nodule (see Table 9.5). Furthermore, many ancillary tests using immunohistochemistry and molecular diagnosis techniques, as mentioned earlier, have been found to be helpful in the diagnosis of equivocal follicular lesions. However, thorough sampling and careful morphologic evaluation still may be the best diagnostic method at present, which can be supported by additional ancillary studies in difficult cases.

Widely Invasive Follicular Carcinoma

The diagnosis of this subtype of follicular carcinoma is fairly straight forward, on gross examination, the tumor has widely invasive edges with foci of necrosis, and 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. These tumors spread to distant organs through blood vessels and up to 80% may develop systemic metastases. The prognosis is much worse than MIFC; the 10-year survival in these patients is 25–45% as opposed to 70–100% in the MIFC group [39, 225]. Various prognostic scoring schemes have been suggested, most including variables such as patient's age, tumor size and extent of invasion, presence of vascular invasion, and metastasis, all of which seem to be important prognostic factors [436].

Oncocytic (Hurthle Cell) Tumors

Thyroid follicular cells with oncocytic features including finely granular abundant eosinophilic cytoplasm were first described by Askanazy in 1898; the cells that were described by Hurthle in the thyroid of a dog are thought to represent the parafollicular C cells [225]. However, the term Hurthle cells for oncocytic thyroid cells that were actually described by Askanazy has been so engrained in our minds that people continue to use this terminology. The AFIP fascicle on thyroid tumors has proposed to designate these tumors as oncocytic tumors and in the recent WHO classification of thyroid tumors these tumors are classified as variants of follicular adenoma and follicular carcinoma [225, 304]. Montone et al. recently reviewed oncocytic lesions of the thyroid that include both neoplastic and non-neolastic types [390]. Oncocytic tumors are a rare group of thyroid neoplasm, with oncocytic (Hurthle cell) carcinoma comprising 3.6% of all thyroid cancers in the USA [201]. While it is derived from

the thyroid follicular cells, oncocytic carcinoma has a distinct oncogenic expression, which is different from the follicular and papillary carcinoma [437]. Oncocytic tumors are divided in to two categories, the benign tumor as adenoma and the malignant counterpart as oncocytic carcinoma [438]. Some earlier studies suggested that all oncocytic (Hurthle cell) neoplasms irrespective of their size have the propensity of distant metastases and should be regarded as carcinoma [439, 440]. This view, however, now is universally not accepted and oncocytic tumors are classified as adenoma or carcinoma on the basis of the capsular and/or vascular invasion criteria identical to the follicular adenoma/carcinoma discussed above.

Oncocytic Adenoma

Oncocytic adenoma is a completely encapsulated tumor with a distinct brown smooth and homogenous cut surface and foci of hemorrhage, and necrosis may be seen as a secondary change. 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 solid or trabecular pattern should raise the suspicion for malignancy and warrants careful evaluation of the capsule [225]. The tumor cell nuclei have a vesicular chromatin and may show significant atypia, hyperchromasia, and pleomorphism, which should not be mistaken as malignancy. 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, which may sometimes involve the entire lesion making histologic evaluation difficult; this is most commonly seen following needle aspiration biopsy [225].

The distinction between oncocytic adenoma and carcinoma is on the basis of the finding of capsular and/or vascular invasion like 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 larger than 4.0 cm being more likely to be malignant compared to the smaller tumors; other immunohistochemical markers such as Ki67 and cyclin D1 expression have also been found to help in the differential diagnosis of oncocytic adenoma and carcinoma [440–443].

Oncocytic Carcinoma

Oncocytic carcinoma is a rare malignant thyroid tumor that is more common in women with a female to male ratio approaching 2:1 which is less than papillary or follicular carcinoma (female to male ratio for oncocytic adenoma is 8:1). While it can occur at any age it is most commonly seen in the elderly, which is a decade later than the oncocytic adenoma [201]. Therefore, an oncocytic tumor in an elderly man, especially if it is larger than 5.0 cm, should raise suspicion of malignancy.

On gross examination oncocytic carcinoma are larger than their benign counterpart, although with a wide size range, and show a brown cut surface with more marked areas of hemorrhage and necrosis and at times may show cystic degeneration in the center. Histological examination most often shows a solid/trabecular growth pattern as opposed to predominantly follicular pattern seen in adenoma and while 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 greater percentage of cells being tall columnar as opposed to round or polygonal seen in adenoma [225]. In addition there are 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 in follicular carcinoma. This distinction is important because of significant difference in the biologic behavior of the two groups. In one study, no patient with minimally invasive carcinoma died of disease after median follow-up of 8 years, while among the widely invasive carcinoma group 73% relapsed and 55% died of disease. Adverse prognostic factors include extrathyroidal extension, nodal metastases, positive margin at surgery, and solid growth pattern. Of these, extrathyroidal extension and nodal metastases are independent predictors of prognosis on a multivariate analyses [438, 444]. Ghossein et al. reported high mitotic rate and solid/trabecular growth pattern is associated with four or more foci of vascular invasion that carried a high risk of local recurrence [445].

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 be distinguished include oncocytic variant of medullary carcinoma, oncocytic variant of PTC, and parathyroid oxyphil type 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. Nuclear changes of PTC can help distinguishing oncocytic tumor from oncocytic variant of PTC. Oncocytic tumors may sometimes exhibit a predominantly papillary architecture comprising of papillae without a well developed fibrovascular core; these tumors are sometimes designated as oncocytic papillary neoplasms

and should be distinguished from oncocytic variant of PTC [225].

Papillary Oncocytic Neoplasms

Oncocytic tumors may sometimes exhibit a predominantly papillary architecture comprising 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 discussed earlier, on the basis of the characteristic nuclear changes of papillary carcinoma.

Poorly Differentiated Carcinoma

Differentiated thyroid carcinoma of both papillary and follicular type may progress to a more poorly differentiated phenotype. Poorly differentiated thyroid carcinoma (PDTC) is a heterogenous group that may be present as more distinct morphologic entity referred to as insular carcinoma or a group of less well-defined morphologic phenotype. The latter group of PDTC, 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 the distinct category in question [446]. In the non-insular group after excluding the aggressive variants of PTC, this group of tumor present as a distinct biological group similar to the insular group so that their classification as PDTC seems justified (see Volante et al. for review [447]). Hiltzik et al. from Memorial Sloan-Kettering Cancer Center defined PDTC on the basis of mitosis (5 or more per 10 HPF) and necrosis and found that these criteria identified a more aggressive subset of thyroid carcinoma independent of the growth pattern [448]. More recently a working group comprising thyroid pathologists from USA, Europe, and Japan at a consensus meeting following review of 83 cases have come up with uniform diagnostic criteria for the diagnosis of PDTC, that include presence of solid/trabecular/insular growth pattern, absence of nuclear features of PTC, and presence of at least one of the following: convoluted nuclei; mitotic activity of 3 or more per 10 HPF; and tumor necrosis; This has come to be known as the "Turin Proposal" [449].

Insular Carcinoma

Insular carcinoma is a morphologically distinct form of PDTC derived from the thyroid follicular cells having an aggressive biological course. The incidence of these tumors varies from being rare in the USA to about 4% of all thyroid cancers seen in an Italian study [225]. It is not uncommon to see foci of insular growth associated with differentiated thyroid carcinoma of both papillary and follicular types. Yamashita et al. in a series of 82 follicular carcinomas reported presence of an insular component in 8 (10%) and furthermore, presence of

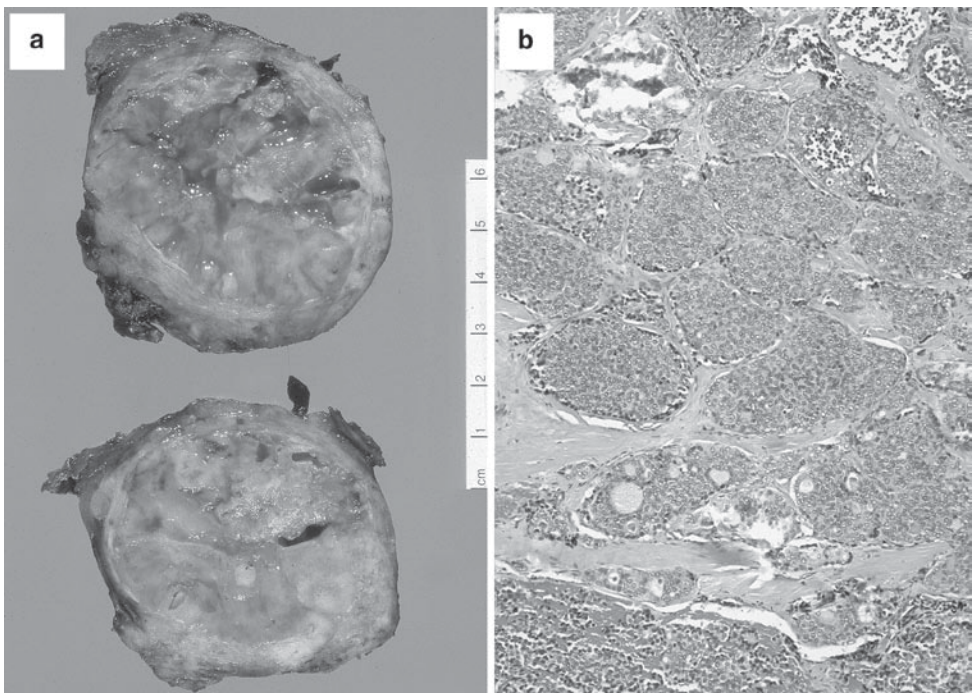

Fig. 9.15 Insular carcinoma: (**a**) macroscopic appearance of thyroid gland with insula carcinoma; (**b**) solid islands of tumor cells with microfollicular structure characteristic of insular carcinoma

insular component was an independent risk factor for distant metastasis [450]. Insular carcinoma tends to involve elderly patients with a slight female predilection.

On gross examination these tumors are large in size and exhibit an invasive growth with associated areas of necrosis (Fig. 9.15a). On histology they are composed of well-defined islands of round or oval cells which lack significant pleomorphism. Small microfollicular structures may be seen within these islands; it is not uncommon to see necrosis in the center of these islands and a peritheliomatous pattern of infiltration by tumor cells (Fig. 9.15b). Foci of vascular invasion are not infrequent and rhabdoid type cells with eosinophilic intracytoplasmic inclusions have also been reported [451].

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 TTF-1 and negative for calcitonin and other neuroendocrine markers. Another primary tumor that may have to be differentiated is the solid variant of papillary carcinoma in which the nuclear features of papillary carcinoma are helpful in the differential diagnosis [396, 397].

PDTC is associated with a prognosis worse than differentiated thyroid carcinoma but better than anaplastic carcinoma. In a series of 49 PDTCs, Jung et al. reported a 5-year survival rate of 68%, which was similar in both insular and non-insular types. In their study adverse prognostic factors on univariate analysis included age 45 years or more, tumor size larger than 4 cm, extra-thyroidal invasion, cervical node metastasis, distant metastasis, absence of high dose radioactive iodine (RAI) therapy, and TNM stage II, III, and IV. However distant metastasis was the only independent prognostic factor [452].

Anaplastic Carcinoma

Anaplastic thyroid carcinoma is a highly aggressive and rare thyroid tumor accounting for 1.7% of all thyroid cancers in the USA [201]. Its incidence is higher in regions endemic for goiter [453]. The patients are usually elderly in their seventh or eighth decade of life with a female preponderance of around 2.5:1. Personal history of goiter may be seen in approximately 25% of cases and prior exposure to radiation in 9.4% cases. Clinical presentation includes 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 [201, 225]. On gross examination the tumor is usually large, majority of such tumors being larger than 4.0 cm, often replacing the entire thyroid and spreading in to the perithyroidal soft tissues. 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 non-keratinizing squamous cell carcinoma, spindle

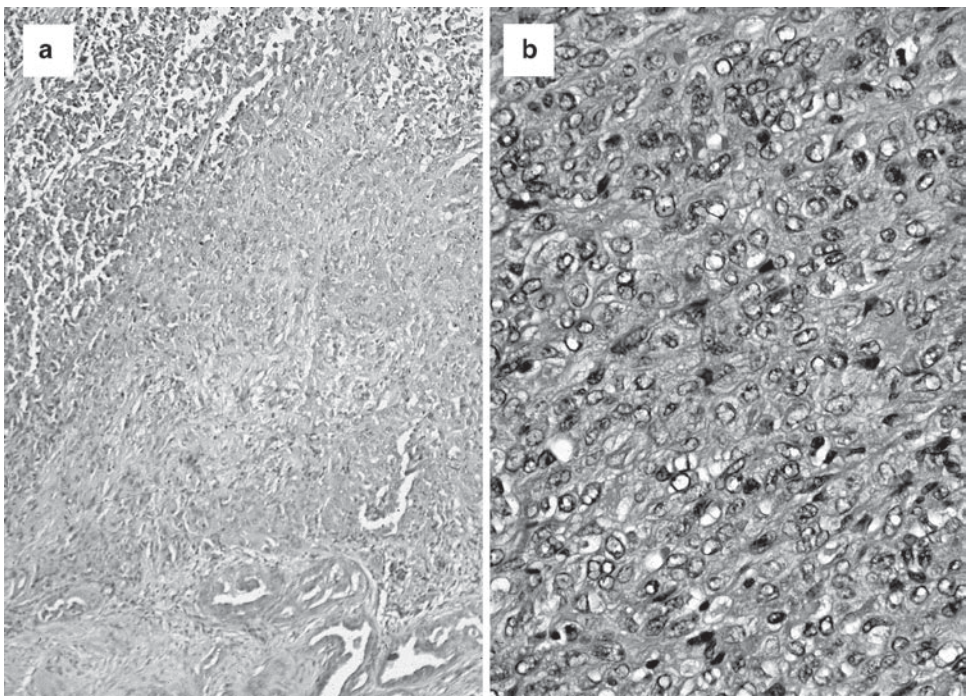

Fig. 9.16 Anaplastic carcinoma: (**a**) giant cell type with necrosis; differentiated papillary carcinoma is seen in the bottom right; (**b**) Spindle cell type

cell pattern resembling sarcoma, and giant cell type (Fig. 9.16). All the three types of pattern are associated with high mitotic rate and focal necrosis may be seen. The spindle cell sarcoma-like pattern may show paucicellular areas with dense fibrosis, which may alternate, with cellular areas, which aid in the diagnosis of anaplastic thyroid carcinoma [39, 225]. The giant cell pattern is associated with marked degree of pleomorphism with large multinucleated cells including tumor giant cells. The tumors are highly invasive with infiltration of the perithyroidal soft tissues. In some cases heterologous stromal elements including cartilage, bone, and rhabdomyoblastic differentiation may be seen [225, 454, 455]. The key to the diagnosis of ATC in most cases is the identification of differentiated thyroid carcinoma that may have progressed to ATC. Albores-Saavedra et al. reported rhabdoid inclusions in PDTC and ATC but not in differentiated component of the tumor, which instead had intracytoplasmic thyroglobulin inclusions suggesting dedifferentiation of PTC and FC to ATC [456]. There is evidence of alteration of certain molecular pathways [225, 457] in the progression of thyroid cancer such as p53 and β-catenin mutations, the latter leading to derangement of E-cadherin/β-catenin complex [458–461]. These studies and others have also provided some insights into potential targets for treating ATC; these targets include EGFR, β-catenin, cyclin E, and cyclin D1 [462, 463].

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; TTF-1 and TTF-2, two other markers crucial for thyroid differentiation, may also be variable and in one study were positive in 18 and 7%, respectively [457]. Ultrastructural evidence of epithelial differentiation is seen in most tumors.

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 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 this differential diagnosis.

The spindle cell type tumor should be differentiated from true *sarcoma* such as *fibrosarcoma, leimyosarcoma, malignant fibrous histiocytoma, angiosarcoma*, and *hemangiopericytoma*. This may pose greatest difficulty especially in cases where the entire tumor is of spindle cell type and is paucicelluar. 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 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 [228–230].

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. 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 [225].

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 are helpful in this differential diagnosis [225].

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 presence of necrosis, more cellular areas, and evidence of vascular invasion and metastases [39]. 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 5-year survival ranging from 0 to 14%, and mean survival being 7.2 ± 10 months [225, 304]. Prognosis is related to extent of disease at presentation and rare cases that do better are tumors localized to thyroid that are less than 5 cm in size and include microscopic foci of anaplastic carcinoma in the background of differentiated thyroid carcinoma [304].

Medullary Thyroid Carcinoma

Clinical Features and Etiology

The tumor derived from the thyroid C cells and now referred to as medullary carcinoma was first regarded as an unusual variant of anaplastic carcinoma [39]. The term medullary carcinoma was coined by Hazard et al. in 1959 who reviewed 600 cases of thyroid cancer over a 31-year period at Cleveland Clinic, reported 21 tumors with unique morphological features including solid growth pattern and amyloid stroma, and called them medullary carcinoma [464]. Later, Williams in 1966 identified the cells of origin of these tumors as C cells

[465]. Medullary thyroid carcinoma (MTC) is a rare tumor and accounts for 5–10% of all thyroid cancers (WHO 2004) [304]. The incidence of MTC was 3.2% (0.5% familial and 2.7% sporadic) of all thyroid cancers in a cohort of more than 5,500 patients in the USA [201]. One of the unique features of this tumor is that it occurs both in a familial setting associated with other endocrine neoplasms as part of the MEN syndrome and also as sporadic tumors in a non-familial setting [466, 467] Sporadic medullary carcinoma is the more common form of tumor representing approximately 80% of all medullary carcinomas. The remaining 20% are familial and are inherited as autosomal dominant trait with high penetrance [39, 201]. The latter may either occur in association with other endocrine tumors as part of MEN IIA, which includes MTC, adrenal medullary hyperplasia-pheochromocytoma, and parathyroid hyperplasia-adenoma and MEN IIB (III) which, in addition, has mucosal ganglioneuroma and skeletal abnormalities. These tumors may also occur as familial non-MEN medullary thyroid carcinoma [468–471]. The gene for MEN IIA and IIB syndrome is called the *RET* protooncogene which is located on chromosome 10. Germline missense mutation of this gene is seen in patients with MEN IIA and MEN IIB [469, 471–474]. Sporadic tumors may sometime show mutation in the *RET* protooncogene [473]. *RET* point mutation may sometimes behave as a dominant oncogene for thyroid follicular cells which may explain association of MTC with papillary thyroid carcinoma in some cases [475, 476].

The age of presentation in the familial form of medullary carcinoma is younger than the sporadic form and both show a slight female predilection. With increased prospective screening of family members of MEN patients, the age of presentation of familial form of medullary carcinoma is becoming progressively younger. 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, carcinoid, and Cushing syndromes [225, 466, 467]. Increased levels of serum calcitonin and CEA are seen in both familial and sporadic medullary carcinoma and are important diagnostic tests. There have been rare case reports with serum calcitonin negative MTC [477].

Pathology of Medullary Carcinoma

The tumors vary in size from barely visible to large tumors, which may replace the entire lobe; the smaller tumors are often seen in patients with MEN II syndrome who undergo prophylactic thyroidectomy for high serum calcitonin levels that are discovered at routine screening. The small tumors that are less than 1.0 cm are referred to as medullary microcarcinoma and are commonly seen at the junction of upper and middle thirds of the lobes [466, 478–480]. In these cases it is important that the entire gland be submitted so that small tumors, which 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 [481].

On microscopic examination medullary carcinoma are usually circumscribed and show a variety of growth patterns which 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 [482]. The most typical growth pattern includes solid nests or trabeculae or 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. Amyloid may be seen in the stroma in up to 80% of tumors, and stains positively with calcitonin on immunohistochemistry (Fig. 9.17c–f). 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 [442, 483]. 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, which is positive in medullary carcinoma [482]. Sex steroid receptors especially progesterone receptor may be seen in medullary carcinoma on immunohistochemistry [484]. Ultrastructurally, the characteristic membrane bound neurosec*ret*ory granules are seen in the cytoplasm.

Medullary carcinoma variants include *follicular* variant, *papillary* variant, *oncocytic* variant, *small cell* variant, *giant cell* variant, *clear cell* variant, *melanotic* variant, *squamous* variant, *encapsulated* variant, and *paraganglioma* like variant [481, 485]. 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 year survival rates are 60–70% and 40–50% respectively. Better prognostic factors include younger age, women, early tumor stage, and familial tumors [486].

C-Cell Hyperplasia and Medullary Microcarcinoma

C cell hyperplasia

General considerations

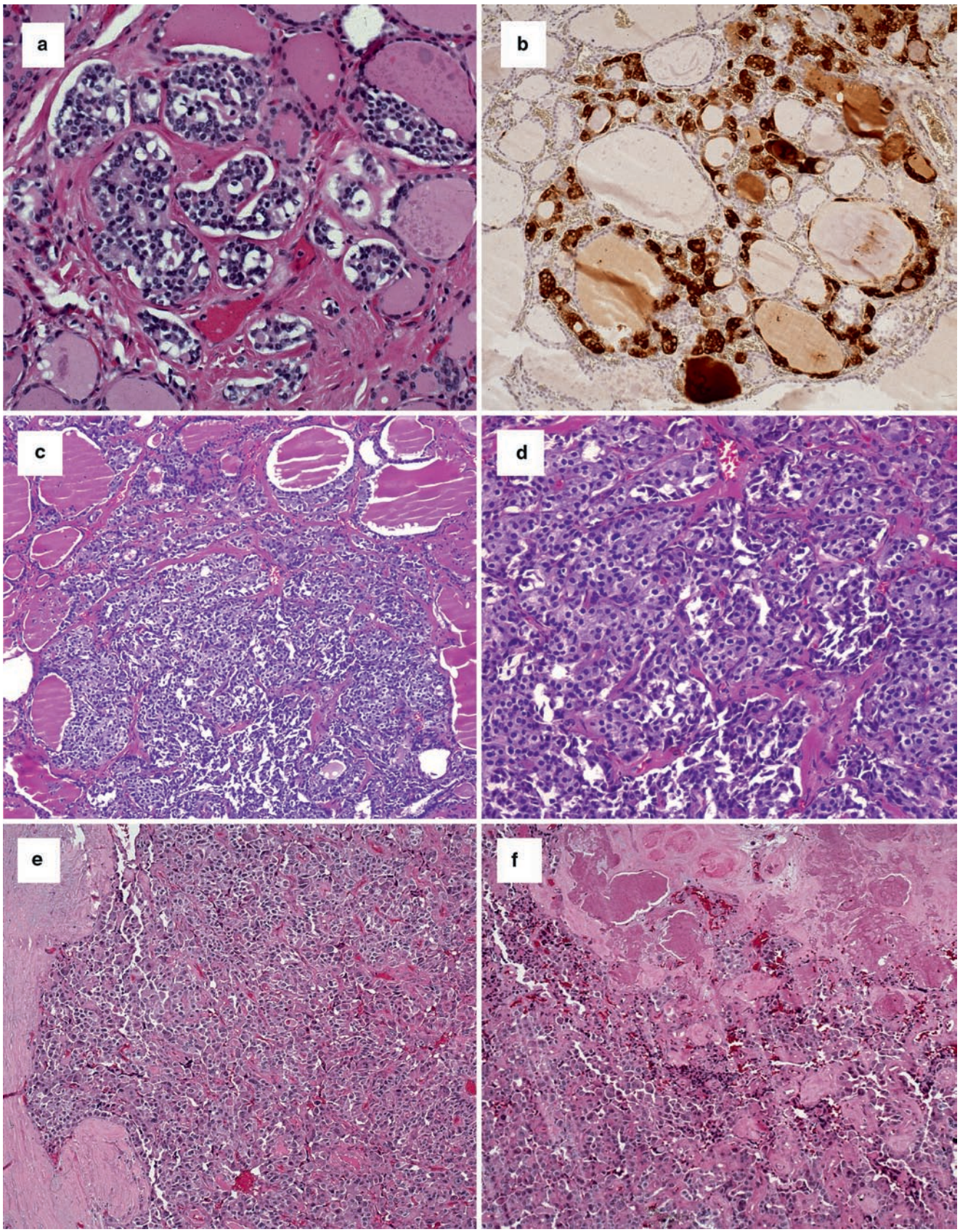

Fig. 9.17 C cell hyperplasia and medullary carcinoma: (**a**) C-cell hyperplasia (Hematoxylin & Eosin); (**b**) C-cell hyperplasia showing positive immunostaining with calcitonin stain (Immunoperoxidase); (**c**) Medullary microcarcinoma in a familial case associated with C-cell hyperplasia; (**d**) same case as (**c**) showing central portion of the medullary microcarcinoma; (**e**) Medullary carcinoma, oncocytic variant; (**f**) same tumor as (**e**) showing amyloid in the stroma

The calcitonin producing C cells are derived from the neural crest and descend down into the thyroid with the ultimobranchial body; therefore, they are mainly found at the junction of the upper and middle third of the two thyroid lobes frequently associated with SCN [18]. C cells are usually visualized on routine Hematoxylin and Eosin stains but may be easily seen on immunohistochemistry. 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 IIA, can also be seen to be associated in certain other situations with no evidence of MEN II. While the former is referred to as familial C cell hyperplasia, thought to be neoplastic proliferation, the latter is called physiological or secondary C cell hyperplasia [487–489].

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 [487, 490]. It is thought to represent the preinvasive stage (carcinoma in situ) of medullary carcinoma [488]. The neoplastic nature of this form of "hyperplasia" is further supported by the fact that the C cells are clonal and show a polysialic acid immunostaining pattern which is distinct from the physiological/reactive C cell hyperplasia [491, 492].

Physiological C cell hyperplasia also referred to as reactive or secondary C cell hyperplasia has been associated with conditions causing hypercalcemia such as hyperparathyroidism [493, 494]. This form of C cell hyperplasia has also been seen associated with Hashimoto's thyroiditis [495, 496], multinodular goiter, hyperthyroidism, and lymphoma [497, 498], around thyroid tumors of follicular cell origin [499], and following subtotal thyroidectomy [500]. The non-familial (sporadic) C-cell hyperplasia usually does not show RET gene mutation and may not be a risk factor for the development of medullary carcinoma [501]. However, others have shown the development of medullary carcinoma in sporadic C-cell hyperplasia and in some of these studies a high serum calcitonin level (>50 pg/ml) is a good indicator of pre-neoplastic potential of reactive C-cell hyperplasia [502].

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 [487, 495], to semiquantitative estimates such as >50 cells per 50 low power fields to >50 cells per one low power field [487]. Guyent et al. have proposed a more precise quantitative criterion which includes >40 cells/cm^2 or more than 50 cells in three low power (×100) fields [503]. We feel that for practical purposes, if one can see C cells on routine Hematoxylin and Eosin stain which are later confirmed as C cells on immunohistochemistry, the lesion should be considered as C cell hyperplasia (Fig. 9.17a, b). In patients with MEN II, 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 extent of follicular involvement and morphology [225]. On immunohistochemistry, C cells stain positively for chromogranin, synaptophysin, calcitonin, CGRP, and CEA.

Differential diagnosis

C cell hyperplasia should be distinguished from SCN, islands of squamous metaplasia which 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.

Medullary microcarcinoma

General considerations

Medullary thyroid microcarcinoma is defined as a tumor less than 1.0 cm in size. Its frequency is directly related to screening of MEN II family members using genetic testing for *RET* protooncogene mutation and is likely to increase with more widespread genetic testing [487]. Medullary microcarcinoma can be seen both in familial setting and as sporadic tumor.

The *sporadic medullary microcarcinomas* 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 [479, 480, 487, 504]. In view of high incidence of lymph node metastases, aggressive surgical management has been proposed in these patients [466]. Adverse prognostic factors include symptomatic cases, high serum calcitonin level, amyloid stroma, and desmoplasia [466, 479].

Familial medullary microcarcinomas are seen in patients showing germline mutation of the *ret* protooncogene. They are detected either on genetic testing, or on finding of high serum calcitonin level. 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 [487]. In patients with MEN 2 prophylactic thyroidectomy is recommended at an early age (<6 years) to prevent progression to medullary microcarcinoma [505–507]. Medullary

microcarcinoma and C-cell hyperplasia found in prophylactic thyroidectomy in at risk patients are frequent but carry a good prognosis [508].

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 [479]. On microscopic examination, the tumors commonly show trabecular, solid, lobular/diffuse growth pattern. Follicular and pseudopapillary patterns 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 [479, 487]. Stromal fibrosis and amyloid deposition may be seen but are usually less than what is seen in sporadic medullary carcinoma larger than 1.0 cm is size. Familial medullary microcarcinomas are usually associated with C cell hyperplasia. 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 [482, 509]. Some authors have used collagen IV immunostaining for evaluation of the basement membrane [510].

Prognosis of medullary microcarcinoma

The prognosis in familial medullary microcarcinoma is much better than larger medullary carcinoma. None of the patients in four series reviewed by Albores-Saavedra and Krueger died of tumor with follow-up being up to 20 years in one of the series [483, 487]. In sporadic medullary microcarcinoma, none of the patients in a series of 34 patients of Kaserer et al. showed evidence of local recurrence or mortality during the mean follow-up of 27 months [466]. However, in another series of 38 patients, two patients died of tumor after 24 and 46 months [479]. Beressi et al. reported a survival rate of 93.9% at 10 years which was significantly greater than what was seen in sporadic medullary carcinoma larger than 1.0 cm [478].

Mixed Medullary and Follicular Carcinoma

These tumors show mixed morphology including both medullary and follicular patterns and mixed immunoreactivity with thyroglobulin and calcitonin [511–513]. 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 GI tract [1, 514]. 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. 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 SCN, follicular cell, 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 [39, 515–519]. 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 [520]; this may be even more important when evaluating cervical lymph nodes involved by the tumor [521]. Other diagnostic pitfalls include nodular tumor like squamous metaplasia that may be associated with fibrosing variant of Hashimoto's thyroiditis [522].

Squamous Cell Carcinoma

Primary squamous cell carcinoma of the thyroid is rare; spindle cell squamous cell carcinoma has been described in association with TCV of papillary carcinoma and is regarded as an unusual type of anaplastic carcinoma with an aggressive behavior [523–525].

Spindle Epithelial Tumor with Thymus-Like Differentiation (SETTLE)

This is a rare thyroid tumor, which predominantly occurs in children and young adults. These tumors are slow growing and present as painless thyroid mass. Local recurrence and distant metastases involving lungs may be seen later in the course of the disease [526–532]. SETTLE is thought to be derived from ectopic thymus or branchial pouch remnant [529, 533]. 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 [527–529]. While most tumors are biphasic, monophasic SETTLE has been reported, which must be distinguished from medullary carcinoma and monophasic synovial sarcoma [534]. On immunohistochemistry, the tumor cells stain positive with cytokeratin and muscle-specific actin, and they are negative for thyroglobulin and calcitonin [528].

Differential diagnosis

Differential diagnosis of thyroid spindle cell lesions include 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 of 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 [39].

Carcinoma Showing Thymus-Like Differentiation (CASTLE)

This is a rare tumor first described by Miyauchi et al. in 1985 and has also been referred to as intrathyroidal thymoma (ITET) [535]. It occurs in the elderly, involves lower or middle third of the gland, and may invade surrounding soft tissues and regional lymph nodes. On histology it shows 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 [532, 536]. On immunohistochemistry tumor cells are positive for CD5 suggesting a thymic origin and they are also positive for cytokeratin and CEA; both thyroglobulin and calcitonin are negative [537]. Differential diagnosis of CASTLE includes metastatic carcinoma, particularly from lung and upper aero-digestive tract. CD5 immunoreactivity is useful in this differential diagnosis as most metastatic carcinomas from the sites mentioned above are CD5 negative [535]. CASTLE without node metastasis has low risk of local recurrence and surgery alone may be sufficient treatment [538]. In a series of 25 cases of CASTLE, Ito et al. reported 5- and 10-year cause-specific survival of 90 and 82%, respectively; nodal metastasis and tumor extension predict a worse prognosis [539].

Familial Thyroid Carcinoma

Familial thyroid cancer can arise from follicular cells (familial NMTC) or from the calcitonin-producing C-cells (familial medullary thyroid carcinoma) and has been recently reviewed by Dotto and Nose and Nose V [540, 541]. Most of the follicular-derived tumors (papillary and follicular thyroid carcinomas) are usually sporadic. There has been great progress in recent years in establishing the molecular genetic defect in these sporadic tumors. Familial forms of follicular-derived neoplasms have been only acknowledged in recent years. Presently, approximately 5% of non-medullary thyroid cancers are considered to be of familial origin. The familial follicular cell-derived tumors or non-medullary thyroid carcinomas (FNMTC) encompass a heterogeneous group of diseases, including diverse syndromic-associated tumors, and non-syndromic tumors.

Table 9.6 Syndromic or familial tumor syndrome with a preponderance of non-thyroidal tumors.

Disorder	Chromosomal location	Gene	Inheritance
Familial adenomatous polyposis (FAP)	5q21	*APC*	AD
PTEN-Hamartoma Tumor Syndrome (PHTS)	10q22-23	*PTEN*	AD
Carney complex	17q24	*PRKAR1a*	AD
Werner syndrome	8p11-21	*WRN*	AR
MEN2A	10q11.2	*RET*	AD

Table 9.7 Non-syndromic or familial tumor syndrome with a preponderance of non-medullary thyroid carcinoma.

Familial papillary thyroid carcinoma with oxyphilia	19p13.2	(TCO) Unknown	AD
Familial papillary thyroid carcinoma without oxyphilia	19p13.2	Unknown	AD
Familial papillary thyroid carcinoma with papillary renal cell neoplasia (fPTC/PRN)	1q21	Unknown	Unknown
Familial papillary thyroid carcinoma (f/PMTC1)	2q21	Unknown	Unknown
Familial multinodular goiter with papillary thyroid carcinoma	14q	Unknown	AD

FNMTC is divided into two groups as summarized in Tables 9.6 and 9.7. The first includes familial syndromes characterized by a predominance of non-thyroidal tumors, such as FAP, *PTEN* hamartoma tumor syndrome (PHTS), Carney complex type 1, and Werner syndrome. In this group there is an increased prevalence of NMTC within a familial cancer syndrome with a preponderance of non-thyroidal tumors (familial tumor syndromes characterized by a preponderance of non-thyroidal tumors). Thyroid neoplasia has been reported with increased frequency in some familial syndromes, such as FAP where the thyroid tumors are papillary carcinoma (most of cribriform-morular variant) and affect about 2% of patients, predominantly women under 30 years of age. In *PTEN* – hamartoma tumor syndrome (PHTS), thyroid neoplasia is the most frequent extra-cutaneous manifestation and over 65% of patients have thyroid disease, including numerous adenomatous nodules, lymphocytic thyroiditis, follicular adenomas, and carcinomas, and less frequently papillary thyroid carcinomas. Other familial

syndromes associated with thyroid neoplasia include, Carney complex, Peutz-Jeghers syndrome, MEN1, MEN2A, and Werner syndrome [540, 541].

The second group includes familial syndromes characterized by a predominance of NMTC, or non-syndromic or familial tumor syndromes characterized by a predominance of NMTC; they are sub classified in different subgroups, such as pure familial (f) PTC with or without oxyphilia, fPTC with papillary renal cell carcinoma, and fPTC with multinodular goiter. Familial NMTC is characterized by three or more first degree relatives with follicular-derived NMTC and occurs regardless of the presence of another familial syndrome [540, 541].

Rare Non-epithelial Thyroid Tumors

Lymphoma

Thyroid lymphoma is rare and accounts for 2.2–2.5% of all lymphomas [39]. It usually occurs in the background of Hashimoto's thyroiditis and is thought to arise from the mucosa associated lymphoid tissue (MALT). There have been rare case reports of primary thyroid lymphoma associated with Graves' disease [542]. 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 [543–545]. Bacon et al. recently described 22 cases of follicular lymphoma of the thyroid which included two distinct groups with different clinical stages at presentation and biological behavior; one group showed t(14;18) translocation and/or expressed Bcl2 and was CD10 positive, while the other lacked Bcl2 expression and was negative for CD10. It may be important to distinguish the two for better management of these patients [546].

Other Non-epithelial Tumors

Mesenchymal tumors, which are more commonly seen in other parts of the body, are rare in thyroid. Primary mesenchymal tumors that have been described in the thyroid include vascular tumors such as cavernous hemangioma [547] and epithelioid hemangioendothelioma [548], granular cell tumor [549], solitary fibrous tumor [413, 550], fibrosarcoma [551], smooth muscle tumors including leiomyoma and leiomyosarcoma [552, 553], osteosarcoma [554], and malignant fibrous histiocytoma [555]. Other tumors and tumor like lesions that have been described in thyroid include Langerhans cell histiocytosis [556], plasma cell granuloma [557], and extramedullary hematopoesis [558]. There are reports in the literature of metastatic uterine sarcoma to the thyroid which have to be differentiated from primary sarcoma [559, 560].

Metastatic Carcinoma

Metastases to thyroid from another primary source are infrequent; tumors that most commonly spread to the thyroid include kidney, breast, and lung carcinoma, but metastasis from hepatocelluar carcinoma has also been reported [225, 561]. Metastatic carcinoma should be considered in the differential diagnosis of poorly differentiated carcinoma especially with a non-insular growth pattern, and clinicopathological correlation and immunostaining with thyroglobulin may be helpful in these situations [562, 563]. When investigating metastatic carcinoma in women it should be kept in mind that thyroid carcinoma can strongly express estrogen and progesterone receptor [564] and may be negative for thyroglobulin on immunohistochemistry; TTF-1 immunostaining along with other clinical and pathological features may be helpful in this situation. There have been reports of collision tumor within the thyroid such as papillary thyroid carcinoma in a metastatic liposarcoma [565].

9.4.3.5 Role of Intra-operative Frozen Sections in the Management of Thyroid Nodules

Intra-operative evaluation of thyroid nodules by frozen section (FS) has been the subject of numerous studies over the years. It was used with a higher frequency in the past when preoperative diagnosis by fine needle aspiration cytology (FNAC) was not widely available making intraoperative evaluation a valuable exercise [566]. The accuracy of FNAC for thyroid malignancy is 90–97% and approaches close to 100% in papillary thyroid carcinoma (PTC) [567–570], on the basis of which definitive surgery can be planned without doing FS. The problem however seems to be the follicular pattern lesions in which the accuracy of both FNAC and FS is lower and almost similar [571, 572]. There are studies in the literature on both sides, some making a case for intraoperative frozen section being a valuable tool in the surgical management of thyroid nodules [573–577], while others argue that FS adds little to the surgical planning in this era of improved preoperative diagnosis of thyroid nodules by FNAC [571, 572, 578, 579]. In two studies, one from Johns Hopkins and the other from Memorial Sloan Kettering, surgical management was altered in less than 5% of cases due to a FS diagnosis [572, 578]. We think that FS is not needed in cases diagnosed as malignant on FNAC because of a high specificity. Secondly, in cases diagnosed as follicular neoplasm or suspicious for follicular neoplasm FS may add little

because of limited sampling during intraoperative evaluation. FS may fail to show capsular or vascular invasion needed for diagnosis of follicular carcinoma, and nuclear changes required for the diagnosis of FVPC may be focal and at times better appreciated in formalin fixed tissue. Furthermore, it is well known that freezing may cause artefactual nuclear clearing leading to a false positive diagnosis of FVPC [580]. One situation where FS may be useful is in the cases where the FNAC is either nondiagnostic/unsatisfactory [574] or suspicious for papillary thyroid carcinoma [570]. In the latter situation, imprint cytology along with FS may be valuable as the nuclear changes of papillary carcinoma are better appreciated on a cytological preparation [566].

In summary the spectrum of thyroid lesions described above, both non-neoplastic and neoplastic in nature, are some of the common specimens encountered in surgical pathology practice and a good understanding of these entities is important for accurate diagnosis.

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Chapter 10
Recent Developments in the Molecular Biology of the Thyroid

Yuri E. Nikiforov

10.1 Introduction

Recent years have been characterized by a significant expansion in our understanding of the molecular biology of the thyroid. It has become clear that the molecular landscape of thyroid papillary carcinoma, the most common type of thyroid cancer, is dominated by mutations that stimulate the mitogen-activated protein kinase (MAPK) pathway. Of these, point mutations of the *BRAF* gene have emerged as the most frequent mutation that can be exploited widely for the diagnosis and prognosis of this cancer type. 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 when preventive surgery is performed solely on the basis of molecular genetic testing.

The progress in molecular biology is expected to penetrate virtually all aspects of thyroid pathology and provide significant help in the diagnosis of thyroid tumors, in the determining of tumor prognosis, and 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, since thyroid follicular cells give rise to malignant tumors with a widely variable biological behavior. Indeed, well-differentiated papillary, follicular, and oncocytic (Hurthle 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 provide the readers with a review of molecular alterations identified in main types of thyroid tumors.

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10.2 Papillary Thyroid Carcinoma

Papillary carcinoma is the most common malignant thyroid tumor, accounting for approximately 80% of all thyroid cancers [1]. It has a distinct propensity for invasion of lymphatic channels, resulting in a high incidence of multi-focal involvement of the thyroid gland and regional cervical lymph node metastases [2]. Distant blood-borne metastases are uncommon (5–10%); approximately 10% of tumors recur locally. Overall, papillary carcinoma has the best prognosis of all thyroid malignancies, with an average of 93% 10-year 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 the patient's death. Many attempts have been made to predict tumor behavior based on microscopic features and more recently on molecular alterations. Most common genetic abnormalities found in papillary carcinoma are mutations activating the MAPK pathway (Fig. 10.1). Of these, point mutations affect the *BRAF* and *RAS* genes, whereas chromosomal rearrangement is a mechanism of activation of the *RET* and *NTRK1* genes. These genetic alterations are found in approximately 70% of all papillary carcinomas, and they rarely coincide in the same tumor [4–6]. This suggests that activation of one of these effectors of the MAPK signaling cascade is sufficient for tumor initiation. However, in addition to their common ability to activate MAPK signaling, each of these mutations is associated with a unique set of differently regulated genes [7] and with distinct clinical and pathologic characteristics of papillary carcinomas (Table 10.1) [8].

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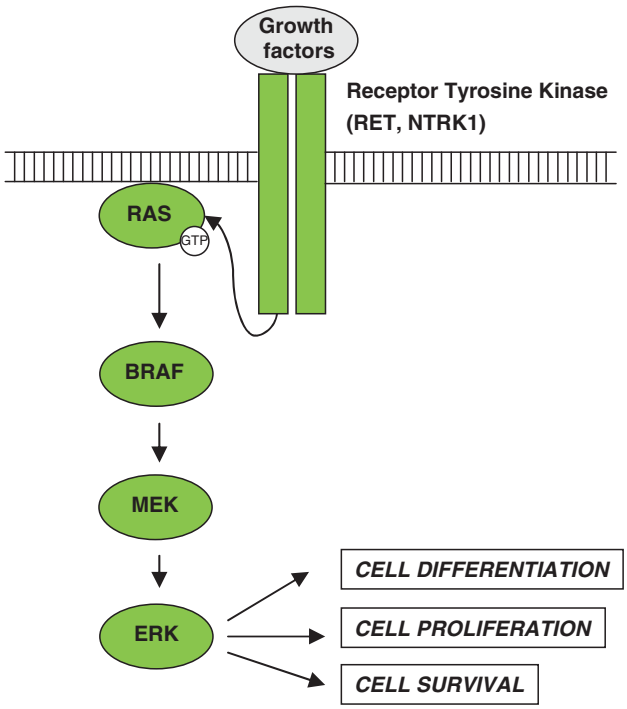

Fig. 10.1 The mitogen-activated protein kinase (MAPK) pathway is frequently activated in papillary thyroid carcinoma cells as a result of point mutation in the *BRAF* and *RAS* genes and chromosomal rearrangement involving the *RET* and *NTRK1* genes. Physiologically, this pathway serves to propagate signals initiated by binding of growth factors to receptor tyrosine kinases, such RET and NTRK1. The activated receptor leads to the activation of RAS located at the inner face of the plasma membrane by substitution of GDP with GTP. The GTP-bound form of RAS recruits BRAF to the plasma membrane and activates it. Activated BRAF activates the mitogen-activated protein kinase/ERK kinase (MEK), which in turn activates the extracellular-signal-regulated kinase (ERK). Once activated, ERK regulates transcription of genes involved in cell differentiation, proliferation, and survival

Table 10.1 Characteristic features of papillary carcinomas with different genetic alterations[a]

	BRAF	*RET/PTC*	*RAS*
Age	Older	Younger	Average
Histopathologic variant	Classic papillary Tall cell variant	Classic papillary Diffuse sclerosing	Follicular variant
Nuclear features	Pronounced	Pronounced	Less pronounced
Psammoma bodies	Common	Very common	Rare
Extrathyroidal extension	More common	Rare	Rare
Lymph node metastasis	Common	Very common	Rare
Tumor stage at presentation	More advanced	Early	Intermediate

[a]Reproduced with permission from: ref. [8]

10.2.1 *BRAF* Mutations

10.2.1.1 Prevalence of *BRAF* mutations in thyroid tumors

Mutations of the *BRAF* gene have been reported in 35–70% of papillary thyroid carcinomas, although in most of the studies the prevalence is close to 45%, making it the most common known genetic event in these tumors [4, 9, 10]. The vast majority of mutations in thyroid cancer involve nucleotide 1799 and result in a valine-to-glutamate substitution at residue 600 (V600E) (Fig. 10.2). Other and rare mechanisms of BRAF activation in thyroid papillary cancer include K601E point mutation, small in-frame insertions or deletions surrounding codon 600 [11–14], and *AKAP9-BRAF* rearrangement, which is more common in papillary carcinomas associated with radiation exposure (Table 10.2) [15]. The V600E and other mutations lead to constitutive activation of BRAF kinase, resulting in continuous phosphorylation of MEK, ERK, and downstream effectors of the MAPK pathway [16].

In thyroid tumors, the V600E *BRAF* mutation is restricted to papillary carcinoma and poorly differentiated and anaplastic carcinomas arising from papillary carcinoma [17–19].

10.2.1.2 Correlation with Microscopic Variants of Papillary Carcinoma

BRAF V600E mutation has a strong association with specific histologic variants of papillary carcinoma. It is more prevalent in the tall cell variant of papillary carcinoma, where it occurs in 70–80% of tumors, and in tumors with classic papillary growth (~60%) [8, 9, 18]. In our experience, BRAF-positive classic papillary carcinomas typically have an invasive border

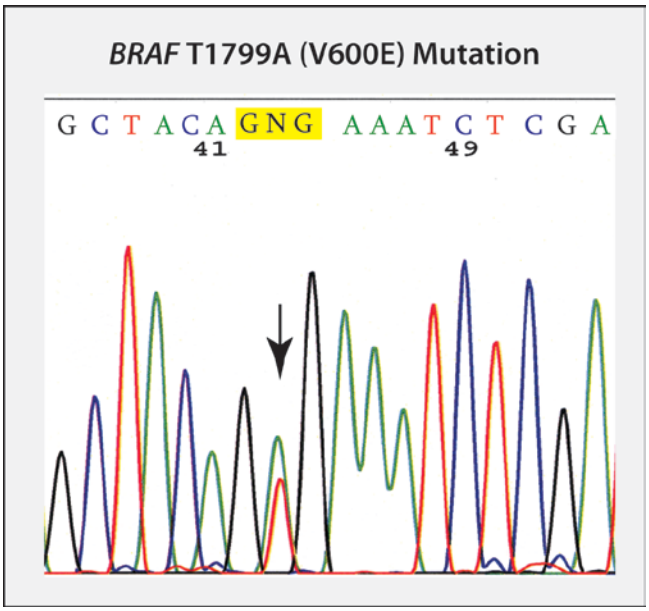

Fig. 10.2 BRAF nucleotide 1799 T→A mutation (V600E) in a papillary thyroid carcinoma detected by PCR amplification and DNA sequencing analysis

Table 10.2 *BRAF* mutations and their occurrence in papillary thyroid carcinomas

Mutation type	Prevalence	Most common histologic variants of PTC
BRAF V600E	40–45%	Tall cell variant, classic papillary carcinoma
BRAF K601E	~3%	Follicular variant
BRAF gene small in-frame insertions or deletions	Rare	N/A[a]
AKAP9/BRAF rearrangement	Rare[b]	Classic papillary carcinoma

[a]Single case reports only
[b]More common (up to 10%) in papillary carcinomas from patients with the history of radiation exposure

and frequently show focal areas composed of tumor cells with tall cell features. On the contrary, *BRAF* V600E is found only in approximately 10% of follicular variants of papillary carcinoma. Another *BRAF* point mutation, K601E, which is overall rare, is mostly found in the follicular variant of papillary carcinoma [20].

10.2.1.3 Correlation with Tumor Behavior

In many studies, the presence of *BRAF* mutation has been shown to correlate with aggressive tumor characteristics, such as extrathyroidal extension, advanced tumor stage at presentation, and lymph node or distant metastases [17, 18, 21, 22]. In two studies, *BRAF* V600E has been found to be an independent predictor of tumor recurrence, even in patients with stage I–II of the disease [21, 23]. A recent study by Elisei et al., which employed a large series of papillary carcinomas with median follow-up of 15 years, demonstrated that *BRAF* V600E mutation was an independent risk factor for tumor-related death [24]. Although the association between *BRAF* mutation and more aggressive tumor behavior has not been found in some studies [20, 25, 26], a large body of literature provides convincing evidence for *BRAF* V600E mutation as a reliable marker of more aggressive behavior of papillary carcinomas.

BRAF activation in thyroid cells appears to alter the subcellular localization and function of sodium iodide symporter (*NIS*) and other genes metabolizing iodide in thyroid follicular cells [21, 27]. This is likely to be responsible for the decreased ability of tumors with *BRAF* mutation to trap radioiodine and treatment failure of the recurrent disease [22]. In addition, *BRAF* association with more invasive growth is likely to be mediated by an overexpression of vascular endothelial growth factor (VEGF), matrix metalloproteinases, and other invasion-promoting protein by mutant *BRAF* [22].

10.2.1.4 Potential Use for Diagnosis and Targeted Therapy

The specificity of V600E *BRAF* mutation among thyroid tumors for papillary carcinoma and poorly differentiated and anaplastic carcinomas arising from papillary carcinoma makes it an attractive diagnostic marker for thyroid surgical and fine needle aspiration (FNA) samples [28]. Indeed, the detection of this mutation in a given thyroid tumor is virtually diagnostic for papillary carcinoma. Among thyroid FNA samples, testing for *BRAF* mutation may be particularly helpful in samples with indeterminate and atypical cytology, as it can help to establish the diagnosis of papillary carcinoma in a significant portion of these aspirates [29–31]. Reliable detection of *BRAF* V600E mutation can be achieved by various molecular techniques using DNA isolated from fresh or fixed FNA samples. In one study, four different detection methods (direct sequencing, colorimetric assay, real-time LightCycler PCR, and allele-specific SYBR green PCR) revealed comparable and high sensitivity of *BRAF* mutation detection in archival FNA smears [32].

Activated *BRAF* is a promising therapeutic target for papillary carcinomas due to high frequency of the mutation and its association with tumor dedifferentiation and resistance to the conventional radioiodine therapy. One of the promising therapeutic agents is BAY 43-9006, a multi-kinase inhibitor with potent activity against RAF and other protein kinases [33]. BAY 43-9006 effectively blocks the wild-type BRAF and the mutant V600E BRAF kinase activity [16, 33]. In preclinical studies, it has been shown to inhibit the BRAF

signaling and growth of all thyroid cancer cell lines carrying the mutant *BRAF* and to impair the growth of the cell line xenografts in nude mice [34]. BAY 43-9006 is being currently tested in clinical trials for several cancer types, including thyroid cancer.

10.2.2 RET/PTC Rearrangement

10.2.2.1 Structure and Function of RET/PTC Oncogenes

Activation of the *RET* gene by rearrangement is the most common genetic event identified so far in papillary carcinomas. The *RET* proto-oncogene is located on chromosome 10q11.2 and encodes a cell membrane receptor tyrosine kinase [35, 36]. 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 [37]. Like other receptor tyrosine kinase, it consists of an extracellular domain, a transmembrane domain, and an intracellular domain that includes a region with protein-tyrosine kinase activity (Fig. 10.3a). The ligands for RET are neurotrophic factors of the glial cell-line-derived neurotrophic factor (GDNF) family [38, 39]. Binding of the ligand 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. 10.3b). Of them, *RET/PTC1* is formed by fusion with the *H4* gene [40], and *RET/PTC3* by fusion with the *NCOA4 (ELE1; RFG)* gene [41, 42]. *RET/PTC1* and *RET/PTC3* are intrachromosomal paracentric inversions since both genes participating in the rearrangement are located on chromosome 10q [43, 44]. 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 [45].

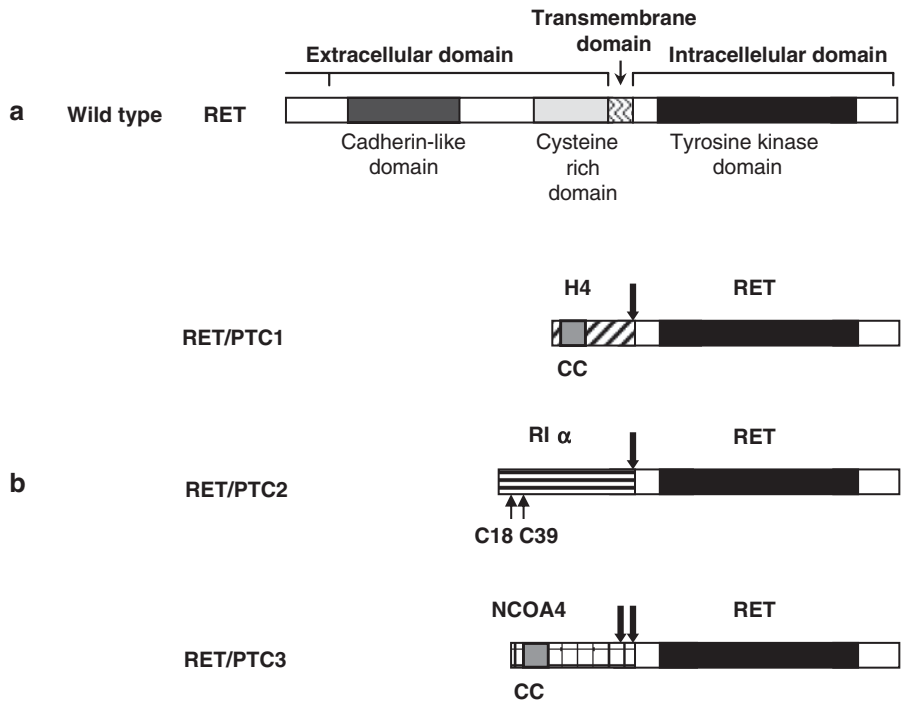

Fig. 10.3 **a** Schematic representation of the wild-type RET gene that encodes a receptor tyrosine kinase. Its extracellular domain contains a region of homology with cadherins and a juxtamembrane cysteine-rich region. The intracellular domain has a region with protein-tyrosine kinase activity, which functions in the phosphorylation of key tyrosine residues and is involved in interaction with downstream targets and activation of MAPK and other 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 NCOA4 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 10.3 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*)	*NCOA4 (RFG, ELE1)*	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	*RFG7*	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/RFG9	*RFG9*	t(10;18)(q11.2;q21-22)	Rare	Yes
RET/PCM1	*PCM1*	t(8;10)(p21-22;q11.2)	Rare	Yes
RET/RFP	*RFP (TRIM27)*	t(6;10)(p21;q11.2)	Rare	Yes
RET/HOOK3	*HOOK3*	t(8;10)(p11.21;q11.2)	Rare	No

More recently, nine novel types of *RET/PTC* have been described (Table 10.3). Most of these rare *RET/PTC* types have been found in papillary carcinomas from patients with a history of exposure to ionizing radiation [45–51], with the exception of *RET/ELKS* and *RET/HOOK3* fusions that have been reported in tumors from patients with no radiation exposure [52, 53]. All of them result from the fusion of the tyrosine kinase domain of RET with the genes located on different chromosomes.

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 which is essential for the activation of the RET tyrosine kinase in the absence of a ligand [54, 55]. In fact, all RET fusion genes encode putative dimerization domains, typically one or more coiled-coil domains [55]. 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 NCOA4 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 NCOA4 protein is distributed [56]. 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.

10.2.2.2 Prevalence in Thyroid Tumors

The prevalence of *RET/PTC* in papillary carcinomas 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% [57–61]. A comparable rate has been reported by other groups with a long-standing interest in the field from Canada (40% [62]) and Italy (29–35%) [59, 63, 64]. In other regions, a wide variation in frequency of RET/PTC has been reported, ranging from 3% in Saudi Arabia [65] to 85% in Australia [66]. Apart from the geographic variability, which clearly exists, some differences are due to the variation in screening techniques and tumor heterogeneity. Most studies reporting a very high incidence of the rearrangement used highly sensitive RT-PCR technique capable of detecting the rearrangement present in only few cells within the tumor, i.e., non-clonal *RET/PTC* [67, 68]. However, when using techniques that detect *RET/PTC* present in a significant proportion of cells within a given tumor (clonal *RET/PTC*), such as Southern blot, fluorescence in situ hybridization (FISH), or standard sensitivity RT-PCR, *RET/PTC* is found with lower frequency, i.e., in 10–20% of adult sporadic papillary carcinomas [67, 69].

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 less than 10% [57, 61, 64].

Even when using similar detection strategies, *RET/PTC* is more common in tumors from patients with a history of radiation exposure and in papillary carcinomas from children and young adults [70–73]. In a series of 92 papillary carcinomas from Italy, 67% of tumors from patients aged 4–19 years harbored *RET/PTC*, in contrast to 32% in those 31–80 years old [63]. In the US series, it was observed in 45–71% of papillary carcinomas from young patients [70, 72].

The prevalence of *RET/PTC* is 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 years after exposure and 49–65% of those removed 7–11 years after the accident harbored *RET/*

PTC [70, 71, 74–76]. Interestingly, *RET/PTC3* was the most common type of tumors developed less than 10 years after exposure, whereas those removed after the longer latency had predominantly *RET/PTC1* [71, 76]. In patients subjected to therapeutic external irradiation for benign or malignant conditions, 52–84% prevalence has been reported [77, 78]. Radiation exposure not only leads to a higher incidence of *RET/PTC* in papillary carcinomas, but also promotes the fusion of *RET* to unusual partners, since 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 [47–49, 79, 80]. The prevalence of RET/PTC in different populations is summarized in Table 10.4.

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 [81] and of fetal human thyroid tissues transplanted into SCID mice [82, 83]. The potential mechanism of how radiation may induce *RET/PTC* has been proposed. It appeared that chromosomal regions participating in *RET/PTC* rearrangements are frequently in close proximity to each other in the nuclei of normal thyroid follicular cells [84, 85]. Such spatial proximity may predispose the two chromosomal regions to simultaneous damage by radiation and facilitate mis-rejoining of free DNA ends located immediately adjacent to each other, which would result in the generation of *RET/PTC*.

RET/PTC rearrangements have been found so far only in thyroid lesions, and are generally believed to be restricted to the papillary type of thyroid carcinoma [86]. 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 [59]. 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 RT-PCR [57].

The presence of *RET/PTC* in follicular adenomas, hyperplastic thyroid nodules, and Hashimoto's thyroiditis, reported in some observations [77, 78, 87–90], has not been confirmed in other studies and remains controversial (reviewed in 69). Overall, it can be assumed that clonal *RET/PTC* (i.e., rearrangement that is found in most cells within the tumor) is reasonably specific for papillary carcinoma.

Two groups have reported the occurrence of *RET/PTC* in hyalinizing trabecular tumors [91–93]. In one observation, four tumors showed RET expression by immunohistochemistry and three of those were found to harbor *RET/PTC1* rearrangement by RT-PCR [91]. In another study, *RET/PTC1* was detected in six out of eight hyalinizing trabecular adenomas by RT-PCR [92]. These findings provide evidence suggesting that hyalinizing trabecular tumors represent a peculiar variant of papillary carcinoma. However, these studies have not demonstrated the occurrence of *RET/PTC* in the majority of cells within these tumors and therefore cannot be admitted as a conclusive demonstration of the association between hyalinizing trabecular tumor and papillary carcinoma.

10.2.2.3 Correlation with Microscopic Features and Variants of Papillary Carcinoma

RET/PTC-positive papillary carcinomas typically present at younger age and have a high rate of lymph node metastases [8]. The rearrangements have been found in papillary carcinomas with classic papillary architecture and in different microscopic variants of the tumor. Overall, this rearrangement, and especially *RET/PTC1* type, appears to be more common in tumors with classic papillary growth and in papillary microcarcinomas (<1 cm in size) [57, 94, 95]. In some studies, the follicular variant of papillary carcinoma had slightly lower prevalence (10–26%) as compared to classic papillary carcinoma (43–47%) [57, 73].

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. 10.4). This finding, originally reported in a series of 38 post-Chernobyl papillary carcinomas [70], has been later confirmed in two larger series of post-Chernobyl tumors [71,96]. It remains unclear, however, if such phenotype–genotype correlation exists in the general population too. It has not been

Table 10.4 Prevalence of *RET/PTC* rearrangements in papillary carcinomas in various populations

		Prevalence (%)	Most common type
Adults, general population		10–40[a]	*RET*/PTC1
Children, general population		50–60	*RET*/PTC1
History of radiation exposure	Post-Chernobyl	60–70[b]	*RET*/PTC3[b]
	External therapeutic irradiation	60–70	*RET*/PTC1

[a]Prevalence widely varies in different geographic areas and is lower when only clonal rearrangement is detected
[b]In tumors developed <10 years after exposure

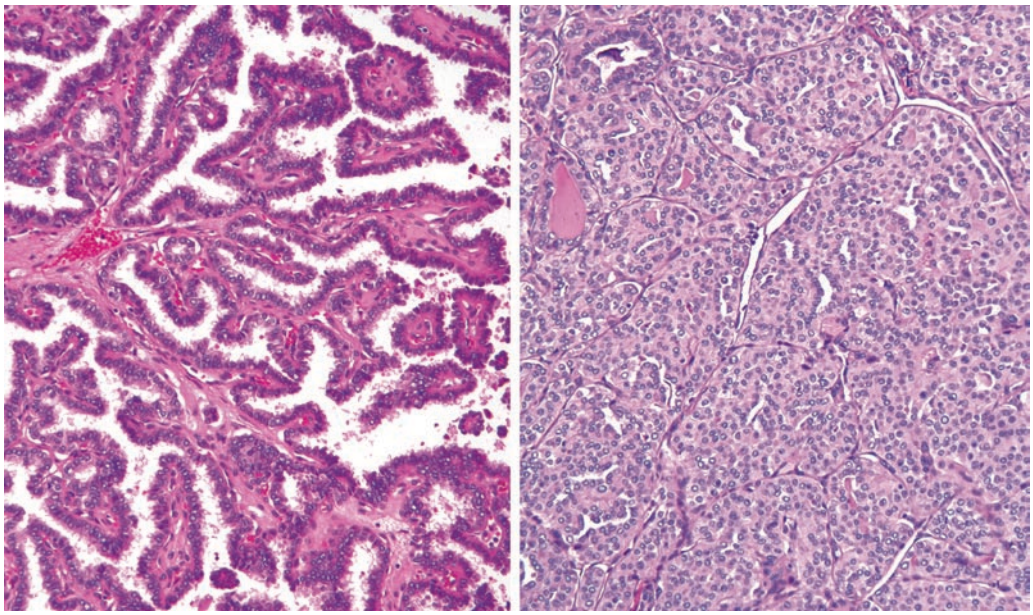

Fig. 10.4 Strong correlation between classic papillary carcinoma (left) and *RET/PTC1* and between solid variant of papillary carcinoma (right) and *RET/PTC3* was observed in post-Chernobyl tumors

found in a recent study of sporadic solid variant and classic papillary carcinomas, although the number of cases analyzed for *RET/PTC* was small [97].

10.2.2.4 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 advanced disease at presentation [64] and distant metastases [58]. Others reported quite opposite findings, suggesting that papillary carcinomas harboring *RET/PTC* have a slow growth and do not progress to poorly differentiated or undifferentiated carcinomas [57, 73, 98]. However, these studies assume that all types of 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 years, no local recurrence or distant metastases was observed in tumors with *RET/PTC1*, whereas those complications were seen in 2 out of 3 carcinomas with *RET/PTC3* [69, 99].
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, while *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 [100]. The cells expressing *RET/PTC3* had a significantly higher proliferative activity, as determined by a fraction of cells in S and G2/M phases of the cell cycle. Moreover, with similar levels of gene expression and tyrosine phosphorylation, *RET/PTC3* transfected cells had 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 non-metastatic thyroid cancers, which do not cause premature death of animals [101–103]. In contrast, transgenic mice expressing *RET/PTC3* develop aggressive and metastatic thyroid tumors [104]. 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 above strongly suggests that it is likely to exist.

10.2.2.5 Potential Use for Diagnosis and Targeted Therapy

RET/PTC rearrangements can be detected in thyroid surgical and FNA samples, and therefore used as a diagnostic marker for papillary carcinoma. In the formalin-fixed surgical samples, the most reliable detection is by FISH (Fig. 10.5). Several studies have shown that *RET/PTC* detection can refine the preoperative diagnosis of thyroid nodules, particularly in samples with indeterminate cytology [29, 105]. Although these studies have provided important evidence for potential diagnostic utility of *RET/PTC* detection in thyroid FNA samples, the performance characteristics of this test have to be defined in a large prospective study before considering the implementation of this test in clinical practice. One potential problem with such a test lies in the requirement for isolation of acceptable quality RNA, which is difficult to achieve in the fixed samples. This limitation can be resolved by collecting a small portion of the aspirated FNA material directly into nucleic acid preservative solution. This approach typically yields sufficient quality and quantity of RNA that can be used for successful *RET/PTC* testing [31].

Activated RET kinase has been explored as a target for therapeutic inhibition by several tyrosine kinase inhibitors in preclinical and clinical studies. ZD6474, an orally active low molecular weight receptor tyrosine kinase inhibitor, is a potent inhibitor of the vascular endothelial growth factor receptor 2 (VEGFR-2) that effectively blocks RET tyrosine kinase [106]. ZD6474 has been shown to block phosphorylation and signaling from RET/PTC3 in vitro, to induce growth arrest of human papillary carcinoma cell lines carrying RET/PTC1 and to prevent growth of *RET/PTC3*-transformed fibroblasts in nude mice [107, 108]. A multi-kinase inhibitor SU12248 (Sunitinib) inhibits signaling from RET/PTC kinase in the experimental models and is currently in phase II clinical trial for radioiodine-refractory unresectable differentiated thyroid cancer [109].

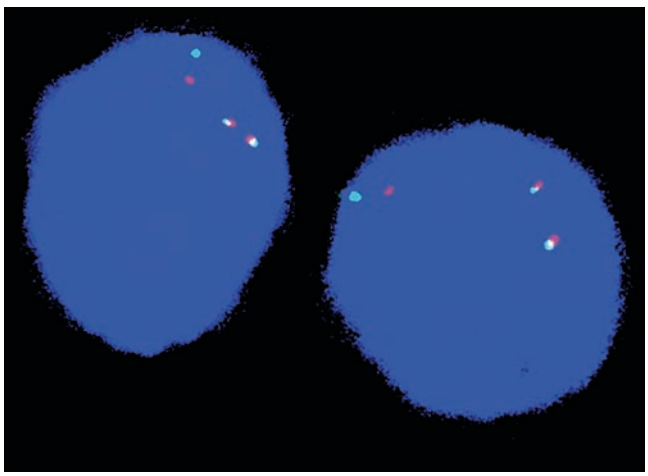

Fig. 10.5 *RET/PTC1* rearrangement in a papillary carcinoma detected by FISH with probes corresponding to the *RET* (green) and *H4* (red) genes. In each cell, a pair of fused signals is present, which indicates the presence of *RET/PTC1*

10.2.3 TRK Rearrangement

TRK rearrangements involving another receptor tyrosine kinase gene, *NTRK1*, also occur in papillary thyroid carcinomas, although at a significantly lower rate. The *NTRK1* gene is located on chromosome 1q22 and encodes one of the receptors for the nerve growth factor [110, 111]. It is expressed in neurons in both peripheral and central nervous system, and is involved in the regulation of growth, differentiation, and survival of these cells. In thyroid follicular cells, the gene is activated through chromosomal rearrangement, which juxtaposes the intracellular tyrosine kinase domain of *NTRK1* to the 5′ terminal sequence of different genes. To date, three fusion partner genes have been identified. Two of them, the tropomyosin (*TPM3*) gene [112, 113] and the translocated promoter region (*TPR*) gene [114, 115], 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 [111]. 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 [116]. Similar to the *RET* fusion partners in *RET/PTC* rearrangement, the genes fused with *NTRK1* are expressed in thyroid follicular cells and drive the expression and chronic ligand-independent activation of the tyrosine kinase domain of NTRK1. *TRK* rearrangement promotes neoplastic transformation of thyroid cells, as has been demonstrated in transgenic mice with thyroid-specific expression of the *TPR/TRK* fusion gene [117]. The mice developed hyperplastic changes in their thyroid glands, and most animals older than 7 months of age had papillary carcinomas.

TRK rearrangements have been reported in up to 10–15% of papillary thyroid carcinomas [64, 111, 118], although in most populations its prevalence is likely to be less than 5%. 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 rearrangement are found with approximately similar frequency, and several tumors with still uncharacterized *TRK* fusion have been reported [64, 118]. The association between *TRK* rearrangement and unfavorable prognosis has been suggested in a study of 119 papillary carcinomas followed on average for 5.7 years [118]. It appeared that tumors carrying *TRK* rearrangement had a

60% local recurrence rate and a 27% tumor-related mortality, both values significantly higher than in tumors without rearrangement.

10.2.4 RAS Mutations

Point mutations of the *RAS* proto-oncogenes (*NRAS*, *KRAS*, and *HRAS*) occur with variable frequency in all types of thyroid follicular cell-derived tumors and are described in more detail later in the chapter. In papillary carcinomas, *RAS* mutations are relatively infrequent, and occur in 10–20% of these tumors [119–126], with the exception of two observations reporting a much higher prevalence (54–67%) [127, 128]. Papillary carcinomas harboring *RAS* mutation almost always have the follicular variant histology; this mutation also correlates with significantly less prominent nuclear features of papillary carcinoma, more frequent encapsulation, and low rate of lymph node metastases [8, 129].

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. In one series of 91 papillary carcinomas, followed on average for 14 years, the incidence of *RAS* mutations was 28% in tumors associated with distant metastases and 8% in tumors confined to the thyroid [123]. However, the role of *RAS* mutation as a marker of aggressive behavior of papillary carcinoma is not fully accepted and awaits further confirmation.

10.3 Follicular Thyroid Tumors

A group of follicular thyroid tumors includes follicular carcinoma, oncocytic (Hurthle cell) carcinoma, and their benign counterparts – follicular adenoma and oncocytic (Hurthle cell) adenoma. These neoplasms originate from thyroid follicular cells and characteristically grow forming variable-sized follicles. Oncocytic (Hurthle) 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 approximately 15% of all thyroid malignancies, with about two-thirds being a conventional type and one-third an oncocytic type carcinoma [3]. It remains unclear whether oncocytic follicular carcinomas and adenomas represent a subset of follicular tumors or are a separate type of thyroid neoplasms 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. Oncocytic 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 FNA specimens. As a result, many follicular lesions are diagnosed as indeterminate or suspicious by FNA cytology and are 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 [130]. This demonstrates the importance of molecular markers that would improve the accuracy of preoperative diagnosis of follicular tumors.

10.3.1 RAS Mutations

10.3.1.1 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, *HRAS*, *KRAS*, and *NRAS*, which encode homologous, but distinct 21 KD proteins. In its inactive state, *RAS* protein is bound to guanosine diphosphate (GDP). After activation, it releases GDP and binds guanosine triphosphate (GTP), initiating signaling through the MAPK and other signaling pathways, such as PI3K/AKT. This pathway eventually leads to transcriptional activation of target genes, which direct the cell to enter the growth cycle. Normally, the activated *RAS* protein becomes quickly 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 activates its downstream signaling pathways.

Table 10.5 Prevalence of *RAS* mutations in thyroid tumors

	Follicular adenoma	Follicular carcinoma	Oncocytic adenoma	Oncocytic 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	[120, 127, 128, 131, 132]	[119, 124, 127, 128, 131, 132]	[133, 134]	[132–134]	[119–124]	[122, 124, 127]	[122, 124, 128, 131, 132]

[a]50% in a subset of microfollicular adenomas [131] and 85% in adenomas from iodine-deficient areas [136]
[b]Two additional studies reported an unusually high prevalence (54–67%) in papillary carcinomas [127, 128]

10.3.1.2 Prevalence 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 originated 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 10.5). Somatic mutation in codons 12/13 and 61 of one of the three *RAS* genes are found in 40–50% of conventional type follicular carcinomas [119, 124, 127, 128, 131, 132] and 20–40% of conventional type follicular adenomas [120, 127, 128, 131, 132]. In adenomas, the mutations appeared to be more common in tumors with a microfollicular growth pattern [131]. A lower incidence has been reported in oncocytic tumors, where only 0–4% of adenomas and 15–25% of carcinomas appeared to be affected [132–134]. *RAS* mutations have also been detected in few cold adenomatous nodules and goiter nodules [120, 132, 135], although it is likely that these lesions are neoplasms and therefore could be better designated as follicular adenomas.

Mutations involving *NRAS* codon 61 and *HRAS* codon 61 are most common, although mutations have been found in different hotspots 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 iodine-deficient areas compared to iodine-sufficient regions [136]. Some early studies of thyroid tumors from experimental animals and humans also suggested the association between *RAS* (especially *KRAS*) mutations and radiation exposure [137, 138]. However, more recent analysis of a large series of follicular and papillary tumors from patients subjected to therapeutic irradiation or accidental environmental irradiation failed to demonstrate such an association [139–141].

10.3.1.3 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 above, they occur in all stages of tumorigenesis including benign follicular adenomas; (2) thyroid-specific expression of mutant KRAS 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 [142]; (3) RAS activation in thyroid cells in vitro results in 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 mutant *HRAS* gene [143]. The overexpression of HRAS resulted in strong stimulation of cell proliferation and extension of their proliferative life span. 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. 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 [144]. 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* are also found 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 chromosome stability. Indeed, in in vitro studies, the mutant RAS has been shown to participate in controlling DNA synthesis and mitosis, and to cause chromosomal instability which manifests as centrosome amplification and chromosome mis-alignment in mitotic cells, leading to chromosome mis-segregation [145, 146]. 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 occur 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 [121, 122, 124]. However, since

these mutations can also be seen in follicular adenomas, 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.

10.3.1.4 Potential Diagnostic Use

RAS mutations are not specific for malignancy and are also found in follicular adenomas. However, they occur with high prevalence in follicular carcinomas and the follicular variant papillary carcinomas, both of which are difficult to diagnose cytologically in thyroid FNA samples. Moreover, since mutant RAS is likely to predispose to progression from follicular adenoma to carcinoma and to further tumor dedifferentiation, it may be justifiable to surgically remove the *RAS*-positive adenomas to prevent such a progression. In one prospective study, aimed to assess the role of detection of different mutations in improving the preoperative FNA diagnosis of thyroid nodules, the detection of *RAS* mutations was found to improve the diagnostic accuracy and allowed to diagnose malignant tumors in several samples with negative or insufficient cytology [31].

10.3.2 PAX8/PPARγ Rearrangement

10.3.2.1 Structure

PAX8/PPAR γ fusion has been recently identified in follicular thyroid tumors with cytogenetically detectable translocation t(2;3)(q13;p25) [147]. One of the involved genes, *PAX8*, is located on chromosome 2q13 and encodes a paired domain transcription factor which plays an important role in thyroid development and differentiation of follicular cell [148, 149]. 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 [150, 151]. 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 [152]. 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. *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 [152, 153]. Both *PPARγ1* and *PPARγ2* are highly expressed in human adipose tissue and at low level in skeletal muscle, while *PPARγ1* has also been found in liver, heart, and some other tissues [152].

In follicular carcinomas with *PAX8/PPARγ* fusion, several transcripts are coexpressed, formed by the fusion of four *PAX8* variants (exons 1–7, 1–8, 1–9, or 1–7 plus 9) to *PPARγ* exons 1–6 [147] (Fig. 10.6). These different *PAX8* variants are apparently a result of the alternate splicing involving exons 8 and 9 known to affect the wild-type *PAX8* [154]. In our experience, the most commonly expressed *PAX8-PPARγ* transcripts in follicular thyroid carcinomas are those containing exons 1–9 and 1–7 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.

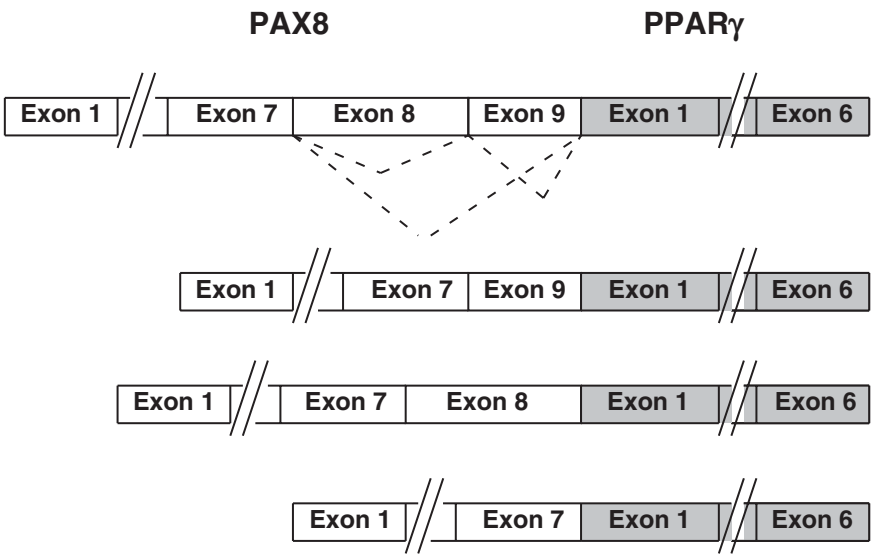

Fig. 10.6 Schematic representation of various PAX8-PPARγ mRNA transcripts coexpressed in follicular carcinomas; they are likely formed by alternate splicing of the PAX8 gene (dashed lines)

10.3.2.2 Prevalence in Thyroid Tumors

PAX8/PPARγ is found in 30–35% of conventional follicular carcinomas, with a lower prevalence in oncocytic (Hurthle cell) carcinomas [155–157]. Tumors that harbor *PAX8/PPARγ* tend to present at a younger age, be smaller in size, have a solid/nested growth pattern, and more frequently reveal vascular invasion [155, 156]. The rearrangement leads to the overexpression of the PPARγ protein that can be detected by immunohistochemistry [147, 158]. However, only strong diffuse nuclear staining correlates with the presence of rearrangement [158].

The rearrangement has also been identified in 2–10% of follicular adenomas [156, 157, 159]. It is conceivable that follicular adenomas positive for *PAX8/PPARγ* are in fact preinvasive (in situ) follicular carcinomas or tumors where invasion was overlooked during histopathologic examination [156]. In support of this possibility, some *PAX8/PPARγ*-positive tumors that meet current diagnostic criteria for follicular adenoma express galectin-3, a beta-galactoside-binding protein typically expressed in malignant thyroid tumors [158].

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 [158]. 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 [160]. 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.

Interestingly, the two most common mutations in follicular carcinomas, *PAX8/PPARγ* rearrangement and *RAS* point mutation, are rarely seen in the same tumor [156]. This suggests that follicular carcinomas may develop via two distinct molecular pathway initiated by either *PAX8/PPARγ* rearrangement or *RAS* mutation.

10.3.2.3 Possible Mechanisms of *PAX8/PPARγ*-induced Transformation

The 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 cell transformation induced by *PAX8/PPARγ* are not fully understood. Some evidence has been presented for inhibition of normal PPARγ function via a dominant-negative effect of the PAX8/PPARγ protein on wild-type PPARγ [147, 161]. Other studies have found the activation of known PPAR target genes in tumors harboring *PAX8/PPARγ*, arguing against the dominant negative effect [162]. Other possible mechanisms include deregulation of PAX8 function, known to be critical for thyroid cell differentiation, and activation of a set of genes related to neither wild-type PPARγ nor wild-type PAX8 pathways [162, 163].

10.3.2.4 Potential Diagnostic Use

The detection of *PAX8/PPARγ* rearrangement may have significant diagnostic value since it occurs mostly in follicular carcinomas. The rearrangement can be detected by RT-PCR, FISH (Fig. 10.7), or immunohistochemistry with PPARγ antibody. The immunohistochemical detection may be challenging to set up and validate since not all commercially available PPARγ antibodies give a reliable result. The identification of *PAX8/PPARγ* rearrangement by RT-PCR or FISH or finding strong diffuse PPARγ inmmunoreactivity in tumor cells during pathological evaluation should justify the submission of additional sections of the tumor capsule and obtaining deeper levels of all suspicious areas in search for capsular or vascular invasion.

10.3.3 PI3K/AKT Pathway Mutations

The phosphatidylinositol-3-kinase (PI3K)/AKT-signaling pathway is an important regulator of cell growth, proliferation, and survival. This pathway can be activated by the

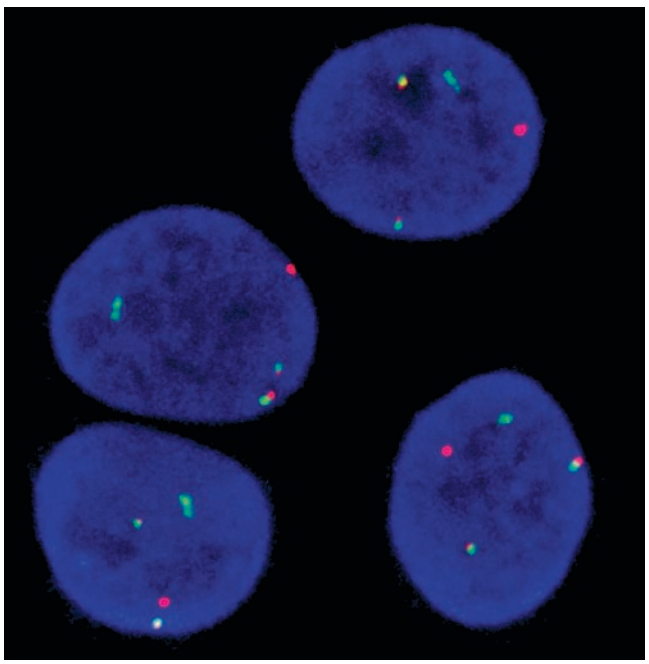

Fig. 10.7 *PAX8/PPARγ* rearrangement in a follicular carcinoma detected by FISH, with probes corresponding to the *PPARγ* (green) and *PAX8* (red) genes. Each cell contains a pair of fused signals, which indicates the presence of *PAX8/PPARγ*.

upstream stimulatory molecules (i.e., RAS), through the loss of function of PTEN protein that normally inhibits PI3K signaling, or as a result of activating mutations or amplification of gene coding for the effectors of this pathway. The *PIK3CA* gene, coding for a catalytic subunit of PI3Ks, has been shown to carry mutations in 6–13% of follicular carcinomas [164–166]. Mutations typically reside in exons 20 and 9 of the *PIK3CA* gene. Mutations of the *PTEN* gene have been reported in a small proportion (~7%) of follicular carcinomas [165, 166].

10.3.4 Loss of Heterozygosity and Other Types of Genomic Instability

10.3.4.1 Loss of Heterozygosity

Loss of heterozygosity (LOH) is characterized by deletions 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 later 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 [167–169]. 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 [167]. 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 [167].

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 [167, 168, 170–173]. Some studies observed that 3p and 17q deletions were significantly higher in follicular carcinomas than in adenomas [170, 174].

Oncocytic (Hurthle cell) tumors, when studied separately, show a comparable or even higher rate of LOH than conventional follicular tumors [171–173]. In oncocytic carcinomas, the most frequently lost regions were on chromosomes 3q and 18q in one study [171], and on chromosomes 1q, 2p, 8q, and 14q in another observation [173]. In the latter report, two markers (1q and 2p) showed a significantly higher rate of LOH in oncocytic carcinomas than in adenomas, with a 100% sensitivity and a 65% specificity in the detection of malignant tumors [173]. Oncocytic adenomas and carcinomas are also characterized by frequent numerical chromosomal abnormalities, including both gains and losses of whole chromosome [134, 175, 176].

It has been suggested that the overall LOH in thyroid tumors more frequently affects the regions that are imprinted, i.e., those with parent-specific expression of genes, as compared to non-imprinted regions [177].

10.3.4.2 DNA Aneuploidy

The pattern of LOH in various types of thyroid tumors generally correlates with the prevalence of DNA aneuploidy, which reflects a loss or gain of whole chromosomes. Thus, less than 10% of papillary carcinoma are aneuploid [178, 179], while 20–30% of follicular adenomas and approximately 50% of follicular carcinomas have aneuploid cell population [180–183]. 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.

10.3.4.3 Microsatellite and Minisatellite Instability

Microsatellite instability is another marker of genomic instability, which manifests as an accumulation of mutations in simple tandem (mono-, di-, tri-, and tetra-nucleotide) 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 oncocytic tumors [172, 184–186]. 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 bp) [187]**.** 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 [183]. The role of minisatellite instability in follicular thyroid tumors is not well understood.

10.3.5 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, TSHR, is a member of seven-transmembrane domain family of peptide 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 the 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 TSHR or any other intermediates along the cAMP pathway will result in TSH-independent stimulation of thyroid cell function.

Indeed, activating point mutations in the *TSHR* gene (located on chromosome 14q31 188) and *GSα* (*GSP*) gene (20q13 [189]) have been found in the hyperfunctioning (toxic) nodules diagnosed as adenomas, adenomatous nodules (which, judged by their clonal origin, were probably true neoplasms [190]), nodules in multi-nodular goiter, and in occasional follicular carcinomas associated with hyperthyroidism. Most studies have reported a 48–82% rate of *TSHR* mutations and a 3–6% rate of *GSα* mutations [191–193], although their prevalence varied markedly in some other series (reviewed in [194]). 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 [195–197]. In both genes, point mutations cluster in the functionally important regions and lead to constitutive activation of the cAMP pathway. In *TSHR*, they are located in the transmembrane domain, involved in the interaction with the G-protein complex, and in the region of the extracellular domain responsible for the receptor affinity to TSH [198] (Fig. 10.8). 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 *TSHR* 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 [199].

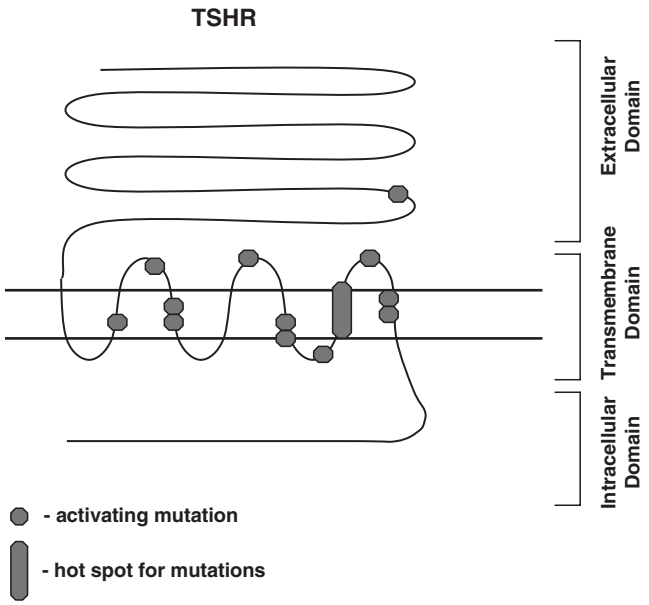

Fig. 10.8 Schematic representation of the TSH receptor and location of activating point mutations in hyperfunctioning thyroid nodules.

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 [200]. Indeed, virtually all animals older than 8 months of age developed thyroid nodules composed of cells demonstrating an elevated cAMP level and high uptake of radioiodine.

10.4 Medullary Thyroid Carcinoma

Medullary carcinoma originates from calcitonin-producing parafollicular, or C-cells and constitutes 3–4% of all thyroid cancers [1, 3, 201]. While most of the tumors are sporadic (non-familial), 15–30% 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 [202–205]. In addition to medullary carcinoma, MEN 2A is also characterized by pheochromocytoma and parathyroid hyperplasia or adenoma, and MEN 2B by pheochromocytoma, neuromas, or ganglioneoromas 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 multi-centric and accompanied by C-cell hyperplasia [206, 207].

10.4.1 RET Point Mutations

Over the last decade, the *RET* proto-oncogene 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 mutations in specific functional regions of the gene are found in almost all patients with familial forms of medullary carcinoma (Fig. 10.9). In MEN 2A and FMTC, they are typically located within the cysteine-rich region in the extracellular domain encoded by exons 10 and 11 [208, 209]. 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 [210–212]. In MEN 2B, more than 90% of mutations involve codon 918 on exon 16 in the intracellular tyrosine kinase domain [211–214]. Exon 15 mutations account for the remaining small percentage of MEN 2B cases [212, 215, 216]. These germline mutations lead to RET activation and conversion into an oncogene through different mechanisms [216–220]. MEN 2A and FTMC mutations replace cysteine with other 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 the 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 [221]. Almost all animals developed bilateral C-cell hyperplasia at as early as 3 weeks of age, and subsequently presented with multi-centric medullary carcinomas.

The discovery of the gene responsible for the familial forms of medullary carcinoma and the availability of reliable tests for *RET* mutations have changed 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 [222–225]. 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 less than 1 cm in size or just a premalignant diffuse C-cell hyperplasia [226].

In sporadic medullary carcinomas, somatic RET mutations are found in 25–70% of cases [212, 227, 228]. 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. Some evidence has been generated suggesting that medullary carcinomas with somatic *RET* mutation have less favorable prognosis, likely due to a higher frequency of regional lymph node and distant metastases as compared to tumors without *RET* mutation [228].

RET kinase represents a potential therapeutic target for sporadic and familial medullary carcinomas. Several RET inhibitors have been evaluated for targeted therapy of medullary carcinomas in preclinical and clinical studies. Two multi-kinase inhibitors that are effective against RET, ZD6474, and SU12248 (Sunitinib), have progressed to phase II clinical trials in patients with familial and sporadic medullary thyroid cancer [106, 109].

10.5 Poorly Differentiated 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

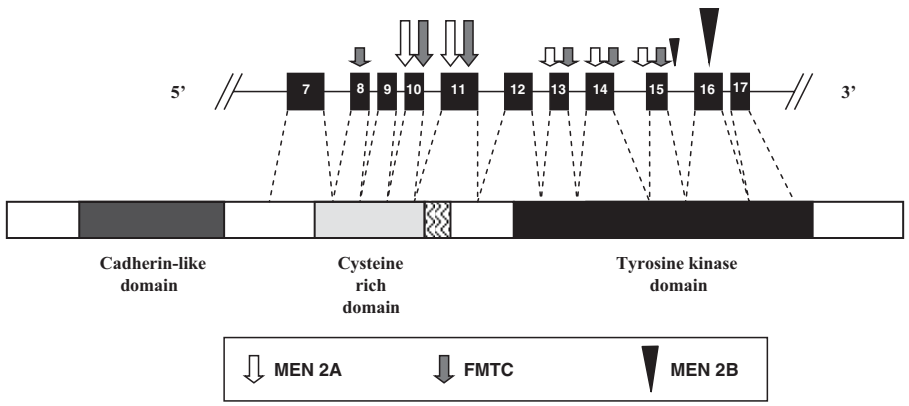

Fig. 10.9 Distribution of germline point mutations in the RET gene and protein in patients with MEN2A, MEN2B, and FMTC. Exons of the gene are represented by boxes and introns by connecting horizontal lines. Larger symbols correspond to a hot spot for mutations in each syndrome

papillary or follicular carcinomas [229]. Anaplastic (undifferentiated) carcinoma represents the most undifferentiated type of thyroid tumors and is one of the most aggressive human neoplasms. It constitutes less that 2% of all thyroid malignancies [3]. In thyroidectomy specimens, both poorly differentiated and anaplastic carcinoma may coincide with the foci of well-differentiated papillary or follicular (conventional or oncocytic type) carcinoma. This indicates that many of them arise from the preexisting well-differentiated tumors following a step-wise progression: well-differentiated carcinoma derived from follicular cells → poorly differentiated carcinoma → anaplastic carcinoma. Such a progression is also supported by a pattern of mutation occurrence in these tumors. Indeed, molecular alterations that are known to be early events in thyroid carcinogenesis, such as mutations of *RAS* and *BRAF*, are found in both, well-differentiated and dedifferentiated tumors or components of the same tumor, whereas other, late events (such as *TP53* mutations) occur only in more dedifferentiated tumors [230].

10.5.1 RAS Mutations

As discussed earlier in the chapter, 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 approximately 60% of anaplastic carcinomas (Table 10.5). The fact that they occur in benign adenomas as well as the experimental data available 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 *TP53* 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 present in both tumor components, whereas *TP53* mutation was found only in the anaplastic carcinoma [231].

10.5.2 BRAF Mutations

BRAF point mutations occur in approximately 15% of poorly differentiated carcinomas and 20% of anaplastic carcinomas, typically in those tumors that also contain areas of well-differentiated papillary carcinoma [17–19, 232]. In these tumors, *BRAF* mutation is detectable in both well-differentiated and poorly differentiated or anaplastic tumor areas. This provides evidence that *BRAF* mutation occurs early in tumorigenesis and predisposes to dedifferentiation. In our experience, many *BRAF*-positive poorly differentiated carcinomas and anaplastic carcinomas contain a well-differentiated papillary carcinoma component that shows microscopic features of tall cell variant.

10.5.3 TP53 Mutations

Point mutations of the *TP53* tumor suppressor gene are among the most common mutations found in human cancer. *TP53* 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. *TP53* becomes overexpressed immediately after the exposure to DNA-damaging agents, such as ionizing radiation and certain chemotheraputic 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 TP53 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 *TP53* 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 well-differentiated follicular and papillary carcinomas [233–237]. Most mutations occur in exons 5 through 8 of the gene and result in alteration of the TP53 DNA binding properties. TP53 inactivation in thyroid cells is not only responsible for accelerated tumor growth, but is also associated with the progressive loss of differentiated markers. Recovery of wild-type TP53 expression in cultured thyroid anaplastic carcinoma cells leads to the reduction in proliferation rate, re-expression of thyroid-specific genes, and re-acquisition of the ability to respond to TSH stimulation [238, 239]. These findings provide evidence that progressive loss of differentiation in poorly differentiated and anaplastic carcinomas is mediated, at least in part, by the inactivation of the *TP53* gene.

Viral TP53 gene therapy aiming to restore the TP53 function has been explored in preclinical and clinical trials for various cancer types, and is under evaluation for anaplastic thyroid carcinoma [240, 241].

10.5.4 β-Catenin (CTNNB1)

β-catenin is a cytoplasmic protein, encoded by the *CTNNB1* gene located on chromosome 3p22–3p21.3 [242, 243]. β-catenin 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) multi-protein 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 *CTNNB1* gene) stabilize the protein by making it insensitive for APC-induced degradation. This results in the accumulation of β-catenin in the nucleus and a 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 [244]).

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 [245, 246]. 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. Since the Wnt-signaling pathway is functionally active in human thyroid cells [247], its constitutive activation by mutated β-catenin is likely to play a role in the progression to poorly differentiated and anaplastic carcinoma.

10.5.5 PI3K/AKT Pathway Mutations

Mutations in the *PIK3CA* and *PTEN* genes affecting the PI3K/Akt-signaling pathway are found more commonly in anaplastic carcinomas than in well-differentiated thyroid cancers. In anaplastic carcinomas, 10–20% of tumors reveal *PIK3CA* mutations and 5–15% *PTEN* mutations [164, 165, 248, 249]. In the *PIK3CA* gene, most mutations are located in exon 20 coding for the kinase domain, and exon 9, coding for the helical domain, and result in the activation of the AKT pathway [164]. In addition, the increase in *PIK3CA* gene copy numbers has been found in about 40% of anaplastic carcinomas [166, 248], although it remains unclear whether one or several extra copies of the gene are functionally sufficient to activate this pathway. In the *PTEN* gene, point mutations and small frameshift deletions most frequently occur in exons 5 and 7 and lead to the loss of function of the PTEN protein and subsequent activation of the AKT pathway [165]. The role of the PI3K/AKT pathway activation in poorly differentiated carcinomas remains largely unknown.

10.6 Summary

Genetic events in various types of thyroid tumors are summarized in the hypothetical scheme of thyroid tumor development and progression on Fig. 10.10.

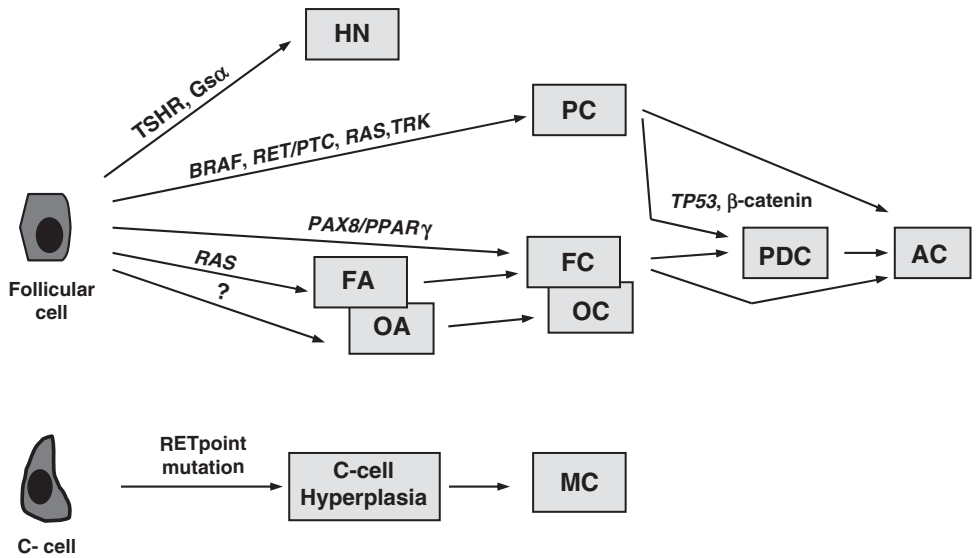

Fig. 10.10 Putative scheme of thyroid tumorigenesis and molecular events in hyperfunctioning nodules (HN), papillary carcinoma (PC), follicular adenoma (FA), follicular carcinoma (FC), oncocytic adenoma (OC), oncocytic carcinoma (OC), poorly differentiated carcinoma (PDC), anaplastic carcinoma (AC), and medullary carcinoma (MC).

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Chapter 11
Adrenal Cortex

Hironobu Sasano, Yasuhiro Nakamura, Takuya Moriya, and Takashi Suzuki

11.1 Historical Overview

The adrenal gland was described nearly 400 years ago, but it was Thomas Addison who provided the first concrete evidence of the vital nature of the adrenal glands through accurate description of the syndrome of adrenocortical insufficiency, termed Addison's disease in 1855. Since then, numerous studies have characterized the biological and biochemical nature of substances produced and secreted by adrenal glands, mainly through the analysis of extracts from adrenals. Kendall at the Mayo Clinic prepared the 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 rheumatoid arthritis in 1949 by Hench and Kendall further enhanced interest in the clinical application of corticosteroids. In contrast to these dramatic improvements and the success of adrenal corticosteroids, the histopathology of adrenocortical diseases had not necessarily kept pace with these advancements of clinical and/or basic endocrinology. Introduction of electron microscopy demonstrated the presence of relatively specific organelles involved in steroidogenesis in adrenocortical parenchymal cells, including mitochondria with specific crista and smooth endoplasmic reticulum. However, these findings could not demonstrate the functional localization of corticosteroids, i.e., which cell types produce what types of corticosteroids, one of the most important aspects of adrenocortical pathology. Introduction of immunohistochemistry led to remarkable improvements in endocrine pathology in general. Antibodies against corticosteroids, mainly developed for radioimmunoassay or enzyme-linked immunoassay, have been employed in the 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 much biologically useful information related to functional pathology.

Corticosteroidogenesis is, in general, catalyzed by specific enzymes, mainly cytochrome P450. Immunohistochemistry of these enzymes, specifically involved in corticosteroidogenesis, could first demonstrate the endocrine-pathological correlation of human adrenal cortex and its disorders [1–9]. In this chapter, histopathological features of major adrenocortical diseases will be described, with emphasis on differential diagnosis and application of immunohistochemical and/or molecular techniques followed by a brief description of the normal adrenal cortex.

11.2 The Normal Adrenal Cortex Histology

Adrenocortical steroidogenesis is under the control of the hypothalamo-pituitary-adrenal axis. It is also important to note that the histology of the "normal" human adrenal cortex changes according to the status of the hypothalamo-pituitary-adrenal axis. In the adrenal of normal subjects, three major zones, zona glomerulosa, zona fasciculata, and zona reticularis, can be appreciated by their histologic structure and their cytologic features [10]. The zona glomerulosa is located 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 clear cortical cells which are arranged in cords or in a column-like fashion. The zona reticularis occupies the inner one-third to one-quarter of the cortex. Cortical cells of the zona reticularis demonstrate eosinophilic compact cytoplasm and are arranged in anastomosing cords [11, 12]. These morphological features of the "normal" or "non-pathological" human adrenal cortex are generally observed in the adrenals of subjects who are not under chronic stress,

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including autopsy specimens after sudden death or in most surgical pathology specimens of adrenalectomy performed concomitantly with nephrectomy for renal cell carcinoma. It is very important for pathologists to recognize the following two morphological features of the adrenals frequently observed in otherwise "non-pathologic" adrenals.

Lipid Depletion: Adrenocorticotrophic hormone (ACTH) is secreted from the anterior pituitary gland in response to stress and causes the adrenal cortex to produce and secrete cortisol. Increased circulating levels of ACTH caused by stress generally results in stimulation of the adrenocortical cells and subsequent lipid depletion of the fasciculata cells following utilization of intracellular cholesterol store [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 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 the nephrectomy described above which demonstrated immunoreactivity of the enzyme mostly in the outer zona fasciculata [1]. Therefore, the post-mortem examination of the adrenal cortex may provide insight into the extent of individual response to ante-mortem stress.

Cortical Nodules: When carefully examining the adult adrenal specimens obtained from autopsy or from surgical resection, some degree of nodularity of the adrenal cortex can be seen in almost all cases. The frequency of these adrenocortical nodules increases with the 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. The adrenal glands with cortical nodules are 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 ante-mortem clinical severity of hypertension and/or hyperlipidemia and/or diabetes mellitus of the patients. Based on these findings, we postulate that adrenocortical nodules occur as a result of localized compensatory overgrowth of adrenocortical cells in response to altered intraadrenal blood flow or localized ischemic changes of cortical cells adjacent to the cortical nodules as shown in Fig. 11.1. Adrenocortical nodules by no means represent neoplastic or pre-neoplastic changes of the adrenal cortex, which is very important for all pathologists, clinicians, and radiologists to recognize when managing patients with incidental adrenocortical mass lesion(s), which will be described later in this chapter. In addition, it is also important to describe these changes when evaluating autopsy adrenal specimens.

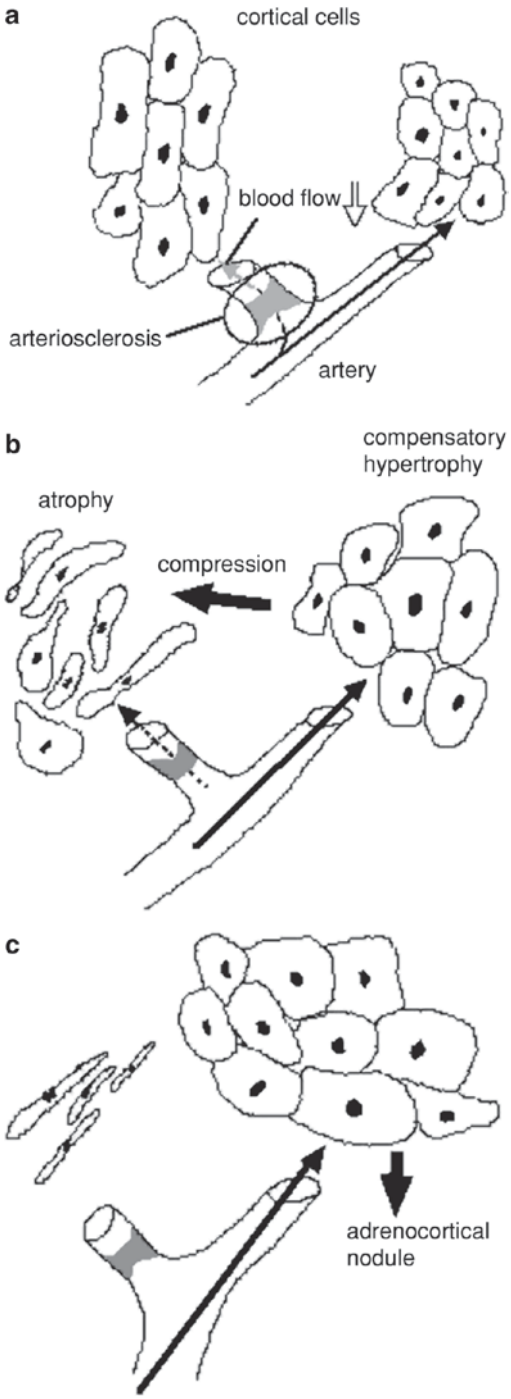

Fig. 11.1 Schematic illustrations of the possible sequence of adrenocortical nodule development. Localized ischemic changes due to atherosclerosis or other causes are considered to result in ischemia and degeneration in the cortical areas supplied by these intraadrenal vessels. In response to these changes, adrenal cortex adjacent to these areas proliferate in a compensatory manner

11.3 Hypercortisolism

The etiology of hypercortisolism can be classified into ACTH-dependent and ACTH-independent. Surgical pathologists rarely receive the adrenal specimens of ACTH-dependent

hypercortisolism due to the recent development and widespread use of transsphenoidal surgery for pituitary ACTH-producing adenomas and other effective conservative medical and/or radiological treatments. Bilateral adrenalectomy is rarely performed in most 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 when receiving the specimens of bilateral adrenalectomy if sufficient clinical and hormonal findings are provided by clinicians. In addition, there have been no established cases which reported that ACTH-dependent hypercortisolism resulted in histologically confirmed functioning of primary adrenocortical neoplasms. Therefore, in this chapter, we will focus on ACTH-independent hypercortisolism.

ACTH-independent hypercortisolism or Cushing's syndrome can be subclassified into neoplastic and non-neoplastic adrenocortical lesions [13]. The great majority of non-neoplastic ACTH-independent hypercortisolism are bilateral but that of neoplastic ACTH-independent hypercortisolism is unilateral. If the resected adrenal glands demonstrated the features of non-neoplastic ACTH-independent hypercortisolism, it is very important to explore the contralateral adrenal gland in order to examine the presence of the disease. It has thus become very important for surgical pathologists to differentiate neoplastic lesions from non-neoplastic lesions in evaluating the resected adrenals from the 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 with asymptomatic cortisol-producing neoplasm that secretes cortisol without clinical evidence of Cushing's syndrome. These cases have been designated as "pre-Cushing's syndrome" or "pre-clinical Cushing's syndrome" [14–16]; however, there are no reported cases of transition from "Pre-Cushing's syndrome" to "full-blown Cushing's syndrome," thereby leading to controversies on using the term "pre-Cushing's" or "pre-clinical." 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 whether the adrenocortical lesions should be resected or not, and if they are scheduled to be removed, it is also important to detect pre-operatively subtle hyperfunctioning adrenocortical neoplasms, especially those 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 will be described.

11.4 Neoplastic ACTH-Independent Hypercortisolism

Among the surgical pathology specimens of ACTH-independent hypercortisolism, a great majority (more than 90%) of the lesions are neoplastic. While studying these specimens in the diagnostic pathology laboratory, it is very important to determine 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, the great majority of Cushing's adenoma demonstrated small and well-circumscribed neoplasm with tan to light-brown color on the cut surface. The majority of tumors demonstrated a heterogenous appearance on the cut surface in the series considered. Tumors with a golden yellow color on the cut surface, as is observed in aldosteronoma are infrequent. 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. The 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 cases presents a homogeneous dark-brown and black appearance, and these adrenocortical tumors are designated as black adenoma (Fig. 11.2). Microscopically, almost all the cortical tumor cells of black adenoma are composed of eosinophilic lipid-sparse compact cytoplasm with many

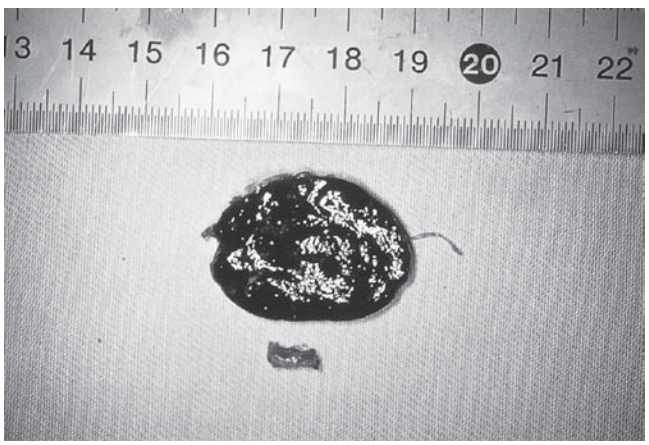

Fig. 11.2 Macroscopic features of black adenoma associated with Cushing's syndrome. Cut surface of the tumor demonstrated relatively homogeneous dark-brown to black appearance. Light microscopic examination generally revealed tumor cells with abundant eosinophilic cytoplasm and lipofuscin granules

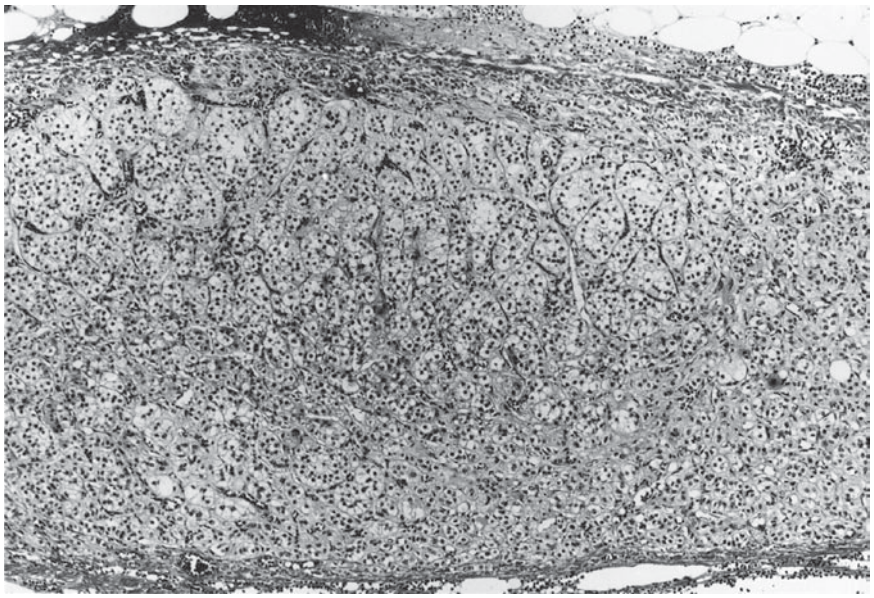

Fig. 11.3 Light microscopic features of attached adrenal in Cushing's adenoma. Both architectural, i.e., thin cortex and cytological cortical atrophy, i.e., marked clear cytoplasm, were detected

lipofuscin in granules. There are no significant differences of serum steroid profiles or secretion patterns of corticosteroids between black and non-black adenomas, and between light-tan and brown Cushing's adenomas [10]. Compact tumor cells generally exhibited much more prominent immunoreactivity of P450c17 [6] and P450c11 [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 the 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. 11.3). The non-neoplastic adrenal may occasionally appear non-atrophic due to the tangential sectioning, but even in these cases cortical cells demonstrated the presence of cellular cortical atrophy, including the clear cytoplasm with abundant lipid and the absence of compact cells (Fig. 11.3). The expression of steroidogenic enzymes, especially that of dehydroepiandrosterone sulfotransferase (DHEA-ST) is markedly diminished in these non-neoplastic attached adrenal cortex [18]. These features 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 non-neoplastic attached adrenal, i.e., pathological findings consistent with ACTH-independent hypercortisolism in the surgical pathology report of patients with Cushing's adenoma.

11.5 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 in molecular and cellular aspects of primary pigmented nodular adrenocortical disease (PPNAD) and ACTH-independent macronodular adrenocortical hyperplasia (AIMAH) which are rare, but are established and important causes of Cushing's syndrome.

11.5.1 Primary Pigmented Nodular Adrenocortical Disease

PPNAD is an unusual cause of Cushing's syndrome first described by Chute et al. in 1949 [19]. PPNAD is clinically characterized by an ACTH-independent bilateral adrenocortical hyperfunction without demonstrable autonomous functioning neoplasm [20–22]. Adrenals of PPNAD are not enlarged and may appear normal in size by CT scan or other diagnostic radiological techniques. In a number of instances, PPNAD has a familial pre-direction with the 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 (CNC)" named after Dr. Carney at the Mayo Clinic. The regulatory R1A subunit of protein kinase A is a key component of the cAMP signaling pathway that has been implicated in endocrine tumorigenesis [23–25]. This gene is localized in 17q22-24 [23, 26, 27]. Heterozygous inactivating germline mutations of PRKAR1A have been demonstrated in about 45–65% of CNC families [23, 27, 28]. LOH at 17q22-24 is observed in tumors from CNC patients, suggesting that PRKAR1A is a tumor-suppressor gene [23]. The frequency of PRKAR1A mutations is about 80% in CNC patients with Cushing's syndrome, suggesting that 17q22-24 defects are more likely to be found in families with PPNAD [23, 29]. 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 Carney complex is transmitted as an autosomal dominant trait but there may be more than one gene involved in this unique disorder. Grossly, the adrenals of PPNAD are very enlarged and ranged from 2.6 to 9.6 g in weight in our series [22]. Multiple tan to black nodules were scattered throughout the cortex on the cut surface of the adrenals 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. 11.4). Immunoreactivity and mRNA in situ hybridization signals (Fig. 11.5) of the steroidogenic enzymes involved in cortisol biosynthesis were detected in almost all of these adrenocortical nodules, in contrast to the adrenocortical nodules in the 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 small sizes of the 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, i.e., suppression of hypothalamo-pituitary-adrenal axis. Immunoreactivity and in situ hybridization signals of steroidogenic enzymes is observed in a small cluster of cortical cells with abundant eosinophilic cytoplasms located at the zona reticularis but not in adjacent non-nodular cortex, which may support an abnormal development of the zona reticularis, and not an exogenous factor, as a possible pathogenesis of the disorders [22].

11.5.2 ACTH-Independent Macronodular Adrenocortical Hyperplasia

In addition to PPNAD described above, increasing number of cases in which bilateral adrenocortical macronodules are observed in the presence of suppressed serum ACTH levels have been recently reported in the literature [30–34]. These adrenocortical lesions were recently designated as AIMAH. AIMAH is clinically characterized by the excessive and autonomous cortisol secretion and the markedly enlarged

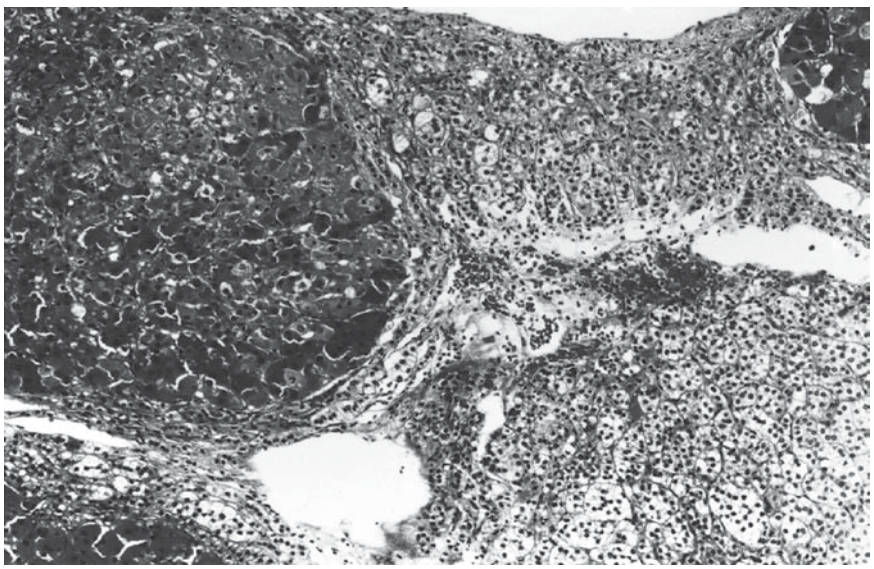

Fig. 11.4 Light microscopic features of PPNAD (primacy pigmented nodular adrenocortical disease). Nodules which are composed of relatively large cortical cells with abundant eosinophilic cytoplasm and adjacent atrophic cortex are observed in this field

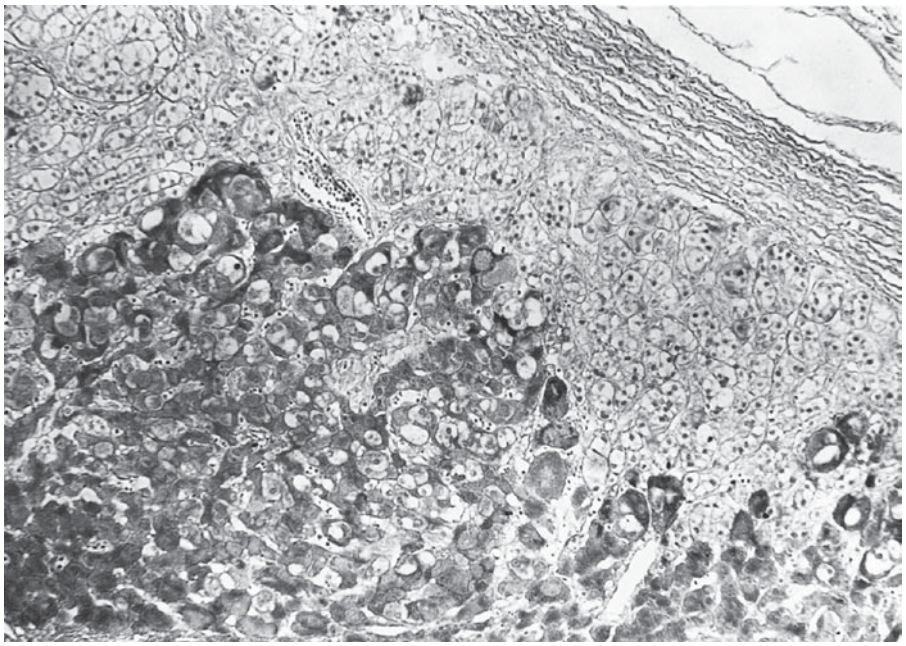

Fig. 11.5 Immunohistochemistry of C17 in the adrenal of PPNAD. C17 or 17alpha-hydroxylase immunoreactivity was predominantly detected in the nodules composed of cortical cells with abundant eosinophilic cytoplasm

bilateral adrenal gland without ACTH hypersecretion. Swords et al. reported a patient with AIMAH in whom an ACTH receptor (MC2R) mutation in the C-terminal of the receptor caused impaired desensitization and internalization of the receptor, and consequently elevated basal cAMP [35]. Swords et al. also described a patient with clinical hypersensitivity to ACTH resulting from two mutations in the same allele of MC2R [36].

Grossly, the adrenal glands are markedly enlarged ranging from 28 to 105 g in our series, and they contain numerous yellow nodules throughout the cortex [34]. Light-tan nodules are also observed in some cases. Normal non-nodular adrenal cortex was generally not discernible. Adrenal glands of AIMAH are composed of two different characteristic cell types, clear and compact cells (Fig. 11.6). Generally, clusters of compact cells are dispersed in the clear cortical cells of the adrenals of AIMAH. Immunoreactivity to P450scc, P450c21, and P450c11 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 hyperplasia11. However, immunoreactivity and mRNA hybridization signals of P450c17 were observed predominantly in small compact cells, while those of 3βHSD occurred exclusively in clear cortical cells [7, 32] (Fig. 11.7). This differential expression of 3βHSD and P450c17 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 P450c17 with cortisol production in the adrenals with AIMAH, but distribution of the enzymes is considered to represent ineffective corticosteroidogenesis, i.e., progesterone produced as a result of 3β-hydroxysteroid dehydrogenation in the large clear cortical cells may not be effectively converted to cortical cells because P450c17 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, 34].

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.

11.6 Adrenocortical Incidentaloma (Hormonally Inactive Adrenocortical Adenoma)

Morphologically, these adrenocortical lesions cannot be differentiated from adrenocortical adenomas associated with Cushing's syndrome or primary aldosteronism [37, 38]. Immunohistochemical analysis of steroidogenic enzymes in these adrenocortical incidentaloma, demonstrated immunoreactivity of all the enzymes involved in corticosteroidogenesis in the tumor cells, except for adrenocortical oncocytoma [37–39]. Adrenocortical oncocytoma is composed of compact cells with abundant lipid-sparse eosinophilic cytoplasms (Fig. 11.8). Electron microscopic examination revealed

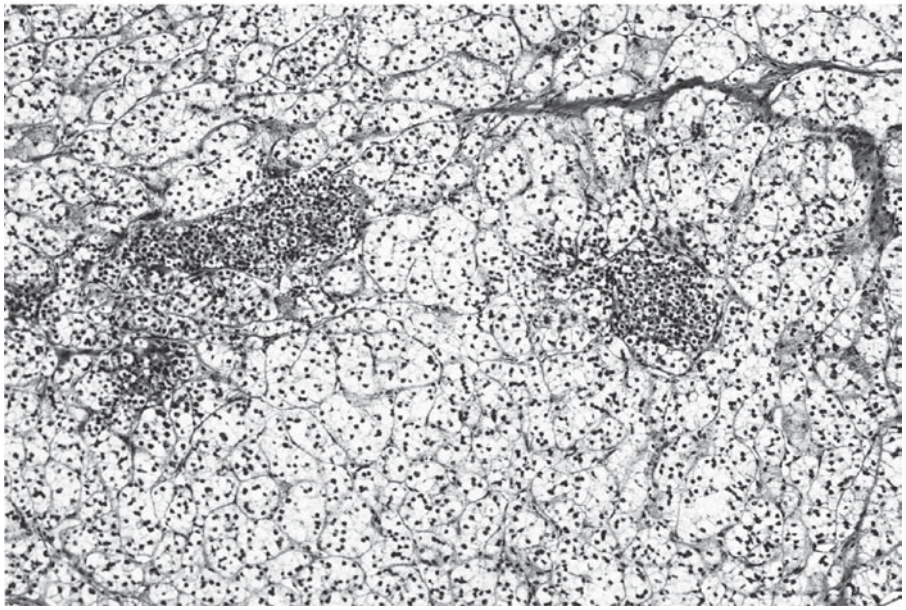

Fig. 11.6 Light microscopic features of AIMAH (ACTH-independent macronodular adrenocortical hyperplasia). Clusters of small cortical cells with scant cytoplasm and relatively large cortical cells with abundant clear cytoplasm were observed in this field

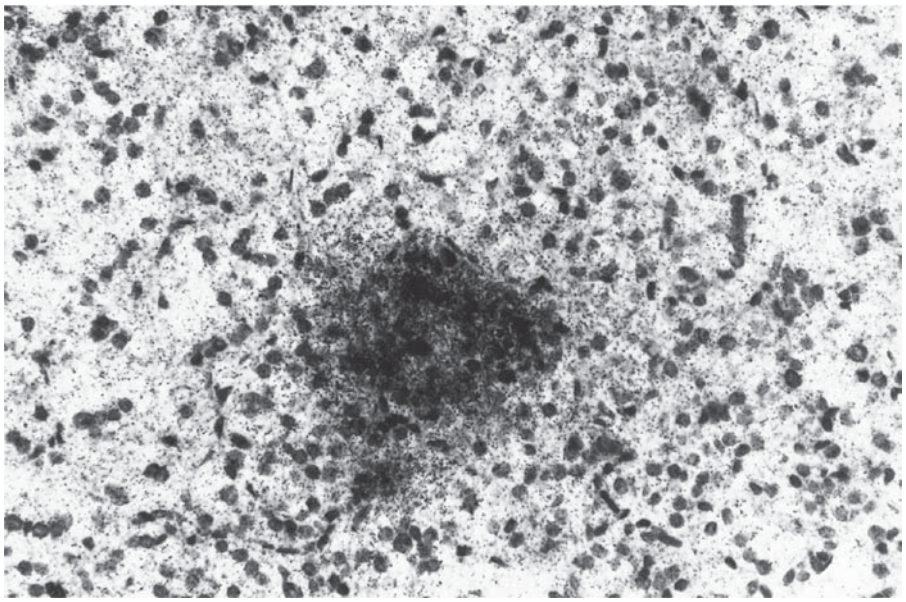

Fig. 11.7 Double immunohistochemistry and mRNA in situ hybridization of C17 in the adrenal of AIMAH. Both immunoreactivity and mRNA hybridization signals were predominantly detected in the compact cells

abundant mitochondria with occasional presence of intramitochondria crystal [39]. These findings indicate that a great majority of incidentally detected adrenocortical lesions can synthesize cortisol, but in insufficient amounts to cause hypercortisolism [37]. The only morphological difference between hormonally active and inactive adrenocortical incidentalomas may be the presence or absence and/or the degree of cortical atrophy of attached non-neoplastic adrenal glands [37, 38]. In addition, the degree of cortical atrophy is to some extent correlated with the degree of suppression of serum cortisol by dexamethasone suppression test and that of diminished expression of steroidogenic enzymes, especially that of DHEA-ST in the attached non-neoplastic adrenal.

Osella et al. reported a correlation between low plasma levels of DHEA-A and increased cortisol non-suppressibility of dexamethasone treatment [31]. Therefore, serum DHEA-S levels are postulated to reflect the status of hypothalamo-pituitary-adrenal axis [40]. The determination of serum

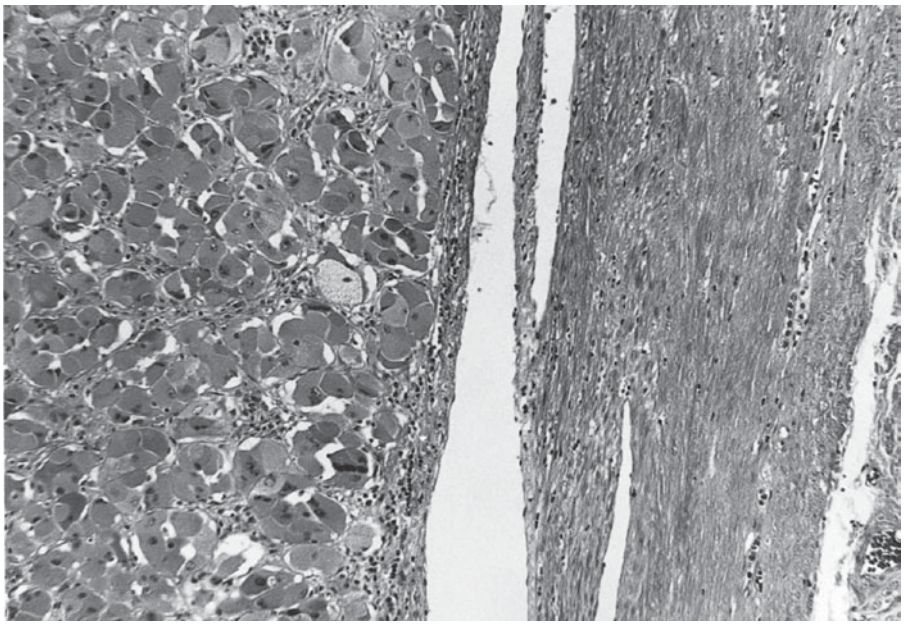

Fig. 11.8 Light microscopic features of an adrenocortical oncocytoma. Relatively large tumor cells with abundant eosinophilic cytoplasm were detected in this field. Compressed non-neoplastic adrenal glands were detected inside the capsule

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 the normal serum levels of DHEA-S decrease with age, and is therefore low in elderly subjects [41, 42]. Therefore, the level of serum DHEA-S levels should be carefully evaluated in the 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 human adrenal is also regulated by ACTH [43]. 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 the attached adrenals 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 the DHEA-ST expression in these adrenals, which can contribute to the more precise evaluation of pre-operative status of hypothalamo-pituitary-adrenal axis of these patients. From a practical standpoint, when confirming that the resected adrenocortical mass is grossly considered as an adrenocortical origin based on the color of the lesion at the cut surface and others and its attached adrenal shows macroscopic adrenocortical atrophy, prophylactic post-operative glucocorticoid replacement therapy is advised for the patients in order to avoid post-operative adrenocortical insufficiency.

11.7 Hyperaldosteronism

Hyperaldosteronism can also be subclassified into primary and secondary hyperaldosteronism based on specific etiology. The pathologists rarely receive the specimens of secondary hyperaldosteronism which are caused by 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 an elevated plasma aldosterone concentration. In patients with primary aldosteronism, the level of plasma aldosterone concentration increased and that of plasma renin concentration suppressed. Primary aldosteronism can also be further subclassified into neoplastic and non-neoplastic primary aldosteronism as in ACTH-independent hypercortisolism. The clinical management of patients, including the requirement of adrenalectomy, is different between neoplastic and preneoplastic hyperaldosteronism as in the patients with hypercortisolism. Therefore, numerous clinical studies have been devoted to hormonal differentiation between neoplastic and non-neoplastic hyperaldosteronism but there has been no single established steroid marker which 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 studies [44, 45]. In addition, we recently experienced one case of unilateral adrenocortical nodule producing excessive aldosterone in our consultation series. However, practically, in patients with primary aldosteronism, it is very important to determine the laterality of the lesions through selective venous sampling and radiological examination, including CT and MRI scans.

11.8 Neoplastic Primary Aldosteronism (Aldosteronoma)

The great majority of neoplastic primary aldosteronism is adenomas and carcinomas are very rare. Aldosteronoma is, in general, a single unilateral well-circumscribed adrenocortical lesion. They may or may not be encapsulated. The size varied from 0.5 to 6 cm in our experience. The great majority of aldosteronoma demonstrated golden-yellow color on the cut surface (Fig. 11.9) but some cases showed light to dark-tan color on the cut surface. Black adenomas associated with primary aldosteronism are very are. Foci of necrosis or hemorrhage are, in principle, not detected. Attached non-neoplastic adrenals may exhibit cortical atrophy, as will be described later in this section. Microscopically, the tumor cells are composed of clear and compact cells. Clear cells can be large or small in size. Expression of steroidogenic enzymes is predominantly observed in the compact tumor cells [12]. Aldosteronoma has been considered to be exclusively involved in aldosterone production. However, dexamethasone suppression tests revealed non-suppressibility of plasma cortisol levels [46]. In addition, P450c17, which is involved in glucocorticoid and/or androgen synthesis but not in aldosterone biosynthesis, is also expressed in compact cells of some tumors [10, 46]. This expression of P450c17 demonstrated autonomous neoplastic production of cortisol, as a result of dexamethasone suppression test and occasional cortical atrophy of zonae fasciculata and reticularis of non-neoplastic adjacent adrenal, showed, due to suppression of pituitary ACTH secretion by excessive autonomous neoplastic cortisol secretion [12, 46]. In the earlier literature, the tumor cells of aldosteronoma were histologically classified as zona glomerulosa, zona fasciculate, and zona reticularis but this classification does not have any inverse biological significance at this juncture, as the study of P450c17 expression demonstrated [12, 46]. 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.

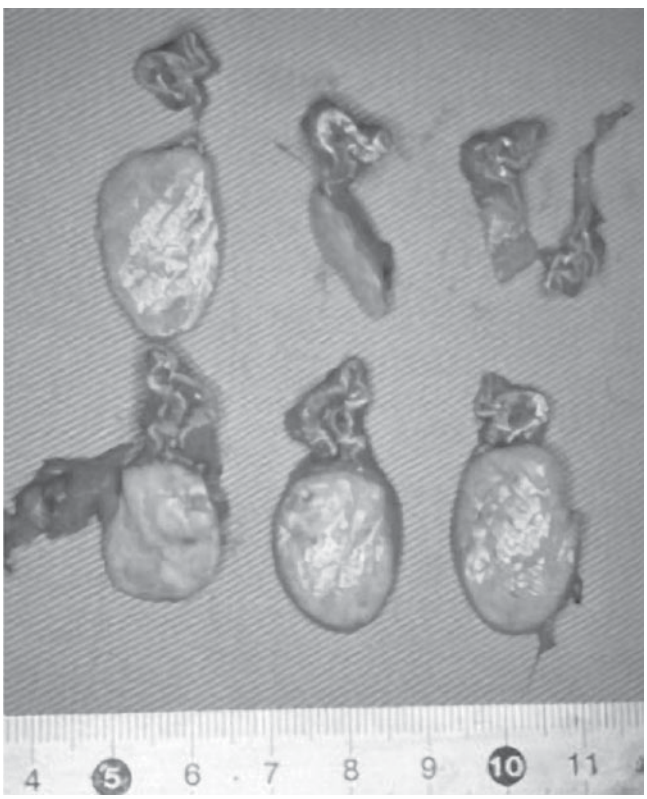

Fig. 11.9 Macroscopic features of an aldosteronoma. The cut surface revealed golden-yellow appearance

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, 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 as paradoxical hyperplasia [11–13]. However, the zona glomerulosa cells in these adrenals did not demonstrate the elevated expression of steroidogenic enzymes except for P450c21 [4, 8]. Therefore, these cells are not considered to be involved in the overproduction of aldosterone. As will be described later, 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 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 a 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 non-familial cases of aldosteronoma, which comprise almost all the cases of aldosteronoma [47]. The abnormalities of this locus, as well as

renin gene restriction on frequent length polymorphism have been proposed as one of the possible basis of genetic abnormalities of aldosteronoma by some investigators [47]. Abnormalities of genomic DNA or the presence of specific gene(s) causing sporadic or non-familial cases of aldosteronoma is so far considered as a remote possibility. Various genetic analyses have failed to identify the characteristic genetic changes associated with aldosteronoma, including the AT1 receptor, the adrenocorticotropin receptor, and natriuretic peptide receptor type A [48–54]. However, it has been recently postulated that the variant 897T of the common functional HERG (human-ether-a-go-go-related gene) encoding for a potassium channel may predispose to the development of aldosteronoma [54]. However, this, of course, needs to be verified by large-scale analysis.

11.9 Non-Neoplastic Primary Aldosteronism

Non-neoplastic primary aldosteronism is subclassified into idiopathic hyperaldosteronism or IHA and dexamethasone suppressible hyperaldosteronism (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 that of aldosteronoma. Less severe hypokalemia, resistance to spironolactone treatment, enhanced aldosterone secretion in response to angiotensin, and others have been reported as characteristics of patients with IHA [13, 55–57]. However, as was 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 [58–60]. DSH is known as an autosomal dominant disease, and molecular mechanisms of this adrenocortical disease has been elegantly clarified by recent investigations [61]. The underlying genetic defect of this disease is the presence of hybrid gene in which 11 beta-hydroxylase gene regulatory elements are fused to the coding region of the aldosterone synthetase gene through unequal meiotic cross-over [62–65]. Therefore, in patients with DSH, this chimeric or hybrid gene encodes a fused P450 protein consisting of the amino-terminal side of 11beta-hydroxylase gene and the carboxyl-terminal side of aldosterone synthetase gene [63]. The expression of this gene is regulated like 11beta-hydroxylase but can also result in the synthesis of aldosterone [62–65]. Therefore, in the 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, molecular and cellular bases of etiology of IHA are not established.

Bilateral or unilateral adrenalectomy was 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 medically treated. The patients with DSH should be treated by glucocorticoid administration. Therefore, it is unlikely for surgical pathologists to examine the adrenal glands of patients who are clinically diagnosed as IHA or DSH. However, it is essential to differentiate IHA, especially those 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 of the cases.

11.9.1 Differentiation Between IHA and Aldosteronoma

As was previously described, it is sometimes difficult to clinically differentiate between IHA and aldosteronoma. This is because of the following reasons: (1) aldosteronoma can be bilateral; (2) the patients of IHA may manifest unilateral macronodule which 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 was 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 long-standing hypertension, which caused nodule formation in the non-neoplastic adrenal in our experience. Histological features of macronodules in the adrenals with IHA can be similar to those of aldosteronoma, i.e., a combination of clear and compact cortical cells. The zona glomerulosa can be morphologically hyperplastic in the adrenals of both IHA and aldosteronoma. Therefore, it is not necessarily easy to differentiate IHA from aldosteronoma even by histological examination in some of the 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 so in those with IHA. 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 P450c17 while those in the non-neoplastic adrenals with aldosteronoma did not have increased expression of the enzymes except for P450c21. It is therefore concluded that immunolocalization of steroidogenic enzymes especially that of 3β-hydroxysteroid dehydrogenase is considered as the only reliable diagnostic method for differentiating IHA from aldosteronoma in the resected adrenals of some patients with primary aldosteronism.

11.10 Diagnosis of Malignancy in Resected Adrenocortical Lesions

When patients with an adrenocortical mass or with an adrenocortical dysfunction are clinically detected, the most important clinical aspects in the management of these patients are whether the adrenal mass represents a malignant neoplasm or whether the 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 which may contribute to differential diagnosis of adrenocortical malignancy will be summarized.

11.11 Macroscopic

When evaluating malignancy in resected adrenocortical neoplasms, macroscopic observation of the specimen submitted to the 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 the adrenocortical neoplasm. In our experience with 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 more than 95 g were malignant, whereas tumors less than 50 g in weight were benign (the average weight of the tumor is 705 g ranging from 96 to 2,460 g) [66]. Van Slooten et al. reported that only tumors with a weight of over 150 g metastasized in their series [67]. However, it is also important to note that small adrenocortical tumor can metastasize while some large tumors cannot. The tumor reported by Gondour and Grizzle weighed only 40 g, and measured only 4 cm in greatest dimension but metastasized 3 years following bilateral adrenalectomy [68]. On the other hand, Hough et al. reported that the tumor weighing 1,800 g did not metastasize [69]. Therefore, the weight of the tumor is important in evaluating the malignancy of adrenocortical neoplasms but the weight itself is not a reliable prognostic indicator of the resected adrenocortical tumor. The next important aspect is the macroscopic feature 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 and hemorrhage, therefore, strongly indicates the diagnosis of adrenocortical carcinoma. However, it is also true that many adrenocortical carcinomas 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 grossing the specimens (Fig. 11.10). It is important to note that foci of intratumoral fibrosis and myxomatous degeneration can be seen in both adenoma and carcinoma, and adrenocortical carcinoma may be well-circumscribed and encapsulated [70]. 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 homogeneous black cut surface, as observed in black pigmented adenoma, is rarely observed in adrenocortical carcinomas.

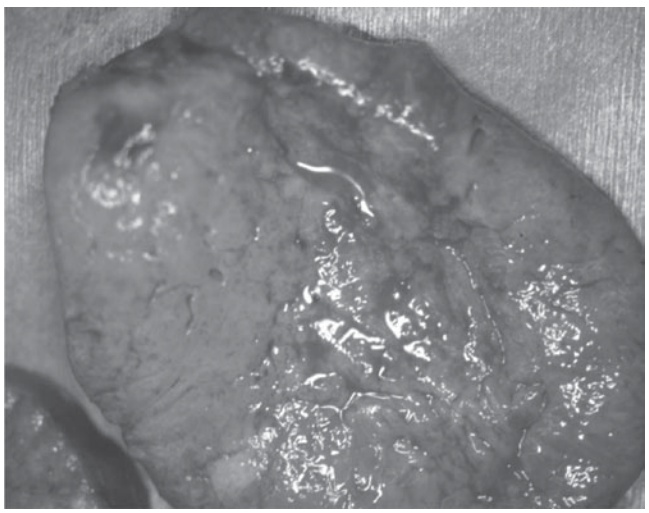

Fig. 11.10 Macroscopic features of adrenocortical carcinoma. Only the specimens sampled from the area adjacent to necrosis and hemorrhage were diagnostic as adrenocortical carcinoma

11.12 Histological Differentiation Between Adrenocortical Adenoma and Carcinoma

It is true that a large number of adrenocortical carcinomas is associated with characteristic gross features described above including large size, necrosis and hemorrhage, and does not usually pose diagnostic problems. However, an increasing number of small adrenocortical neoplasm has been discovered with the development of CT and MRI scans. Therefore, adrenocortical carcinomas not associated with these omnious macroscopic features have recently increased in number. The distinction of this "well-differentiated" adrenocortical carcinoma from adenoma may be one of the most diagnostic difficulties in surgical pathology practice. There are no single histological criteria which can reliably differentiate adrenocortical carcinoma from adenoma-like capsular or vascular invasion of thyroid follicular carcinoma. Only the systems which evaluated multiple histological and/or non-histological criteria of the resected cases can provide reliable histological diagnosis. Three different histological scoring systems have been proposed by various investigators, and all systems are equally useful for predicting clinical outcome of patients with resected adrenocortical neoplasms [67, 69, 71, 72]. They are summarized in Tables 11.1–11.3. Hough and his colleagues proposed 12 criteria, seven histologic and five non-histologic, for predicting the clinical outcome of patients by studying 41 cases of adrenocortical tumors [69]. A numerical value for these 12 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 non-histologic indices were subsequently determined. The combination of histologic and non-histologic 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 familiar with the system. 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 11.2), with assigned numerical value for differentiating adrenocortical carcinoma from adenoma [67]. The numeric values for these severe histologic parameters described in Table 11.2 were subsequently combined and a histologic index for the case obtained. A histologic index of more than eight is considered to correlate with aggressive biological behaviors. When we retrospectively applied these criteria of van Slooten to resected adrenocortical tumor, some adrenocortical adenomas are 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 11.3), which is important in evaluating the adrenocortical malignancy [71]. 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 [72]. The system is straightforward and relatively easy to use, and a good correlation can be detected between results and clinical outcome of the patients.

Table 11.1 Weiss system.

1. High nuclear grade
2. Mitotic figures more than 5/50 hpf
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 11.2 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. Mitotic activity (more than 2/10 high power field)	9.0
7. Vascular or capsular invasion	3.3

Table 11.3 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 1/10 high power field)	0.60
7. Pleomorphism	0.39

Non-histologic Criteria	Numeric value
1. Tumor mass (more than 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 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 months)	2.00

However, it is also true that the tumors which did not behave in a malignant fashion in their post-operative course, including the cases of adrenocortical oncocytoma, were considered as adrenocortical carcinoma, although these adrenocortical oncocytomas may recur or metastasize in a long period of time [39]. In addition, among these nine criteria, we experienced that nuclear grade, architecture, and cytoplasm were likely to be subjective, i.e., 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 these histological scoring systems in order to diagnose the 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 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 tumors 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 neoplasm.

11.13 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 to 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 pre-malignant conditions in 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 carcinoma is markedly heterogenous in morphology and biological function even among 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 the 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.

11.13.1 DNA Content

Adrenocortical neoplasms that recurred or metastasized were reported to likely demonstrate DNA aneuploidy than those showing no evidence of further disease during the post-operative follow-up period [73]. We also reported that seven of eight adrenocortical carcinomas demonstrated DNA aneuploidy while all adenomas were diploid by flow cytometry [74]. However, a number of studies also reported that 20–40% of adrenocortical adenomas have DNA aneuploidy and a small subset of carcinoma was diploid [75–77]. 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 [78]. Therefore, the value of DNA ploidy in determining the biological behavior of resected adrenocortical behavior is still in dispute and further studies are required to establish it as a possible auxiliary mean of evaluating adrenocortical neoplasms.

11.13.2 Cell Proliferation

Cell kinetic information is becoming a valuable adjunct to histopathologically based tumor classification [79]. 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 Ki67 is considered to recognize a nuclear antigen present in all phases of the cell cycle while the PCNA is an auxiliary protein of DNA polymerase delta and is associated with the late G1 and S-phase of the cell cycle. The availability of MIB-1, in combination with antigen-retrieval, made it possible to perform Ki67 immunostaining in 10% formalin-fixed and paraffin-embedded materials (Fig. 11.11). The Ki67 labeling index of adrenocortical carcinoma was reported to be significantly higher than adrenocortical adenoma by our group [79] and the group of Lloyd [80]. On the other hand, PCNA labeling index was not significantly different between adrenocortical adenoma and carcinoma, as was reported in other human malignancies [74, 80]. In our study of immunohistochemical evaluation of Ki67 in human

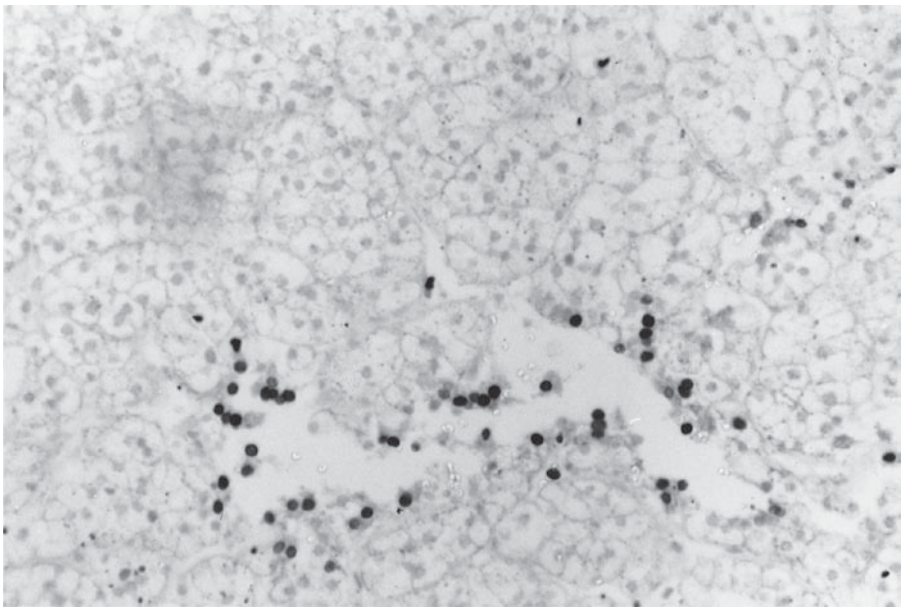

Fig. 11.11 Immunohistochemistry of Ki67 in an adrenocortical carcinoma

adrenocortical neoplasms, 11 of 17 carcinomas demonstrated a labeling index of more than 2.5 whereas none of the adenomas did [81]. Therefore, resected adrenocortical neoplasms of LI with more than 2.5 may represent adrenocortical carcinoma. Therefore, Ki67 immunostaining is of importance in the differentiation between adrenocortical adenoma and carcinoma and may be incorporated in 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 Ki67 LI to surgical pathology differential diagnosis between adrenocortical adenoma and carcinoma. These differences can be frequently experienced through evaluation of Ki67 immunostain of resected neoplasms in various laboratories. This is due to (1) the uneven distribution of Ki67 immunoreactivity, i.e., how many fields are necessary to count, and (2) the interpretation of weak nuclear immunoreactivity, i.e., the threshold of positivity when we apply Ki67 LI to resected adrenocortical carcinoma. In our laboratory, we select at least 10 fields and count at least 500, preferably 1,000 tumor cells. Even when we use computer image analyzer for the evaluation of Ki67 immunohistochemistry, the selection of the fields for counting and the threshold of nuclear positivity can still be a problem.

11.13.3 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 in their possible roles in modifying corticosteroids 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. Previously, overexpression of transforming growth factor α and epidermal growth factor receptor was demonstrated in adrenocortical carcinoma cases [82]. Elevated expression of insulin-like growth factor (IGF) II was reported in the functioning of adrenocortical carcinoma [83]. Very recently, Wilkin et al. demonstrated that induction of the phenotype of the fetal adrenal cortex by IGF-II overexpression and steroidogenesis as well as defective apoptosis may be the cause of pediatric adrenocortical tumors [84]. IGF-I overexpression was also reported in human adrenocortical carcinoma [85]. Inhibins and activins are dimeric proteins of the transforming growth factor-beta super family. They were demonstrated to be present in human adrenal cortex and its disorders [86–88]. Munro et al. recently reported that loss of inhibin alpha subunit may be involved in the progression of adrenocortical carcinoma [86]. Fetsch et al. reported that immunocytochemistry of anti-alpha inhibin can reliably differentiate between adrenocortical and renal cell carcinoma in the specimens of fine needle aspiration [87]. However, Arola et al. reported no significant differences of inhibin alpha expression between benign and malignant adrenocortical tumors and further investigations are required for clarifying the possible roles of inhibins/activins in the development and progression of adrenocortical neoplasms [88]. 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 [89]. Boccuzzi et al. also reported that the association between TGF-beta 1 expression and active steroid secretion is lost in adrenocortical carcinoma [90]. Murray et al. demonstrated the decrement of alpha 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 [91].

Vascular endothelial growth factor (VEGF) plays an important role in the regulation of tumor angiogenesis that is critical for tumor growth and metastasis [92]. A previous study reported that VEGF levels are significantly lower in adrenocortical adenoma than in adrenocortical carcinomas [93].

11.13.4 Cytogenetics

Etiology or the mechanism of tumorigenesis of human adrenocortical carcinoma is unknown but it appears that susceptibility to adrenocortical carcinoma appears to be inherited in some individuals or families.

Children with Beckwith-Wiedemann syndrome, a very rare growth disorder characterized by macroglossia, gigantism, and omphalocele have an increased incidence of a number of tumors, including adrenal adenomas and adrenocortical carcinomas [94–97]. Genetic abnormalities affect the chromosome region (11p15) which contains several genes including IGF-II, CDKN1C (cyclin-dependent kinase inhibitor $p57^{kip2}$) and H19 (an untranslated mRNA) [98, 99]. The $p57^{kip2}$ gene is an embryonic cyclin-dependent kinase inhibitor that acts to negatively regulate cell proliferation and actively direct differentiation [99]. H19 gene encodes a 2.3-kb non-coding mRNA which is also strongly expressed during embryogenesis [99]. Genomic imprinting is a normal embryonic/fetal process which involves methylation of DNA regions leading to stable patterns of transcriptional gene activation or silencing [99]. Within this locus, IGF-II is normally expressed from the paternal allele due to imprinting of the maternal copy, while CDKN1C and H19 are paternally imprinted and maternally expressed [99, 100]. Individuals with Beckwith-Wiedemann syndrome often have uniparental paternal isodisomy (a form of LOH), i.e., loss of maternal locus with an accompanying gain of paternal allele, resulting in a remarkable overexpression of IGF-II with concomitant decrease in $p57^{kip2}$ and H19 expression [99]. In addition, adrenocortical carcinoma is part of a constellation of tumors inherited in the sarcoma, breast, lung, and adrenocortical carcinoma syndrome described by Li and Fraumeni and Lynch et al., called Li–Fraumeni syndrome, which is also very rare in occurrence [101, 102]. The p53 gene, located on the 17p13.1 chromosomal segment, encodes the 393 amino acid tumor suppressor protein situated in the center of a network of signaling pathways that are essential for cell growth regulation and apoptosis induced by a diverse array of cellular stresses [99]. In a patient with Li–Fraumeni syndrome, tumors arising from germline loss of p53 include breast cancer, soft tissue sarcomas, brain tumors, osteosarcoma, leukemia, and adrenocortical carcinoma [99, 103]. These observations suggest that some carcinoma, although small in number, are considered occurring as a result of spontaneous transformation of adrenocortical cells by spontaneous mutations of genomic DNA.

Various studies have suggested that the 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 neoplasm [104]. 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 [104]. A breakpoint of 11p13, as well as loss of heterozygosity of alleles on 11p15, has been recently reported in adrenocortical carcinoma cases [105]. 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 [106]. Both adenomas and carcinomas demonstrated chromosomal imbalances but several chromosomal gains, especially high-level amplifications, were almost exclusively detected in adrenocortical carcinoma. Zhao also reported that the most frequent DNA copy changes in adrenocortical carcinomas were the losses of 1p21-31, 2q, 3p, 3q, 6q, 9p and 11q14-qter as well as the gains of 17p, 17q, and 9q32-qter in their CAH study [107]. These authors postulated that oncogenes determining the early tumorigenesis of adrenocortical tumors may exist on chromosome 17 [107]. 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 [108]. These inconsistent results of CAH analysis reemphasized the heterogeneity of human adrenocortical neoplasm.

Previous germline mutations of the p53 tumor suppressor gene have been implicated in the etiology of this disorder [109]. The germline mutations detected in Li–Fraumeni syndrome appears 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 [110]. Subsequent studies revealed that germline p53 mutations were also found in cancer-prone individuals which were not otherwise indicative of the Li–Fraumeni syndrome [109, 111]. 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. reported

that three of six children with adrenocortical carcinoma were found to carry germline p53 mutations in exons 5, 6, and 7, respectively [112]. Barzon et al. recently reported that mutations in the p53 gene are frequent in adrenocortical carcinomas [113]. However, it is also important to note that the patients with adrenocortical carcinoma, with the possible exception of a specific pediatric case, 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 the cases with sporadic adult adrenocortical carcinoma are considered not to harbor germline mutations of p53 but it awaits further investigations 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 i.e., three out of 11 cases demonstrated p53 abnormalities, although none of the five adrenocortical cases demonstrated p53 abnormalities [114]. McNicol et al. reported that abnormal p53 expression did not appear to have any significant prognostic effects in carcinoma [115]. We also could not detect any p53 abnormalities, including the 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 an important role 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 c-myc oncogene product in adrenocortical carcinoma but it awaits further investigations to clarify the practical importance of these findings [74]. Gortz et al. reported that inactivating mutations of the MEN-1 tumor-suppressor gene appear not to play any role in the development of sporadic adrenocortical neoplasms [116]. Heppner et al. also reported that the majority of seven adrenocortical carcinoma cases examined were associated with 11q13 loss of heterogeneity, in which MEN1 gene is located, but somatic MEN-1 mutation within the MEN-1-coding region was a rare event [117]. Nakazumi et al. reported the possible involvement of decreased expression of p27, a cell cycle inhibitor, in the biological behavior of adrenocortical neoplasms [118]. Pilon et al. demonstrated an important role of inactivation of the p16 tumor suppression gene in the pathogenesis of human adrenocortical tumor [119]. Hirano et al. reported the possible correlation between telomerase activity and biological behavior of adrenocortical neoplasms [120]. Mantovani et al. recently reported that the four regulatory subunits (R1A, R1B, R2A, R2B) of protein kinase A were highly expressed in the adrenal carcinoma than the normal adrenal cortex [121]. Babinska et al. demonstrated the statistically significant correlation between p53, p21, PCNA or Ki67 and the occurrence of metastases in adrenocarcinoma [122]. Barzon et al. reported that the expression levels of ERα and aromatase are significantly higher and ERβ level was lower in adrenal carcinoma compared to the normal adrenal cortex [123]. Results of these studies employing molecular and cellular biological tools pointed to the importance of the 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 the biological behavior of resected adrenocortical neoplasms but little has been examined in this field. Further investigations in this field may contribute greatly to our understanding of adrenocortical neoplasms.

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Chapter 12
Adrenal Medulla and Paraganglia

Anne Marie McNicol

12.1 Introduction

Paraganglia are neuroendocrine organs mainly comprising cells that take their origin in the neural crest. They secrete catecholamines or indolamines and peptides. They are divided into two groups, associated with the sympathetic or parasympathetic nervous systems. Sympathetic paraganglia lie close to the paravertebral and prevertebral ganglia in the para-axial region of the trunk or in the connective tissue adjacent to the pelvic organs. The largest is the adrenal medulla. They secrete catecholamines in response to sympathetic neural stimulation. Parasympathetic paraganglia lie close to vascular structures and branches of the glossopharyngeal and vagus nerves in the head and neck. They include the carotid body. They function as chemoreceptors responding to changes in oxygen pressure in the arterial blood.

The main tumors arising from these organs are paragangliomas. Historically, these have been given a range of names. Sympathetic paragangliomas have been referred to as pheochromocytoma; parasympathetic have been named chemodectomas, glomus tumors, or nonchromaffin paragangliomas. The current approach defined by the World Health Organization (WHO) in 2004 [1] is to reserve the term "pheochromocytoma" for intra-adrenal tumors and to define the others as paragangliomas of sympathetic or parasympathetic type, further defined by site.

In recent years, there have been a number of very interesting developments in the genetic aspects of paragangliomas, leading to the understanding that about 30% of them have a genetic basis, rather than the 10% figure taught historically.

The WHO classification [1] also changed the definition of malignancy, limiting it to the presence of metastases, and defining local invasion separately. There are still problems for the pathologist in predicting malignant behavior in primary tumors.

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This chapter outlines some of the relevant aspects of development, anatomy, and function of paraganglia. There is a general discussion on tumors and separate sections on pheochromocytoma, and extra-adrenal sympathetic and parasympathetic paragangliomas. Genetic aspects are also included where relevant, but are covered in detail elsewhere.

12.2 Historical Aspects

The concept of a paraganglionic system was first put forward by Kohn [2] at the beginning of the twentieth century. He also invented the term "chromaffin reaction" for the brown color change that occurred in the adrenal medulla on immersion in chromate salts and "chromaffin cells" for the cells that underwent this change. He described deposits of chromaffin tissue in the retroperitoneum outside the adrenal gland and confirmed the finding that the carotid body contained some chromaffin cells [3]. He proposed that all these tissues were linked to the sympathetic nervous system; resembled, but were not, ganglia; and should be termed paraganglia. The demonstration that the innervation of the carotid body is parasympathetic and that most of the cells are negative for the chromaffin reaction challenged the concept. This led to the modification in which paraganglia were subtyped as chromaffin (associated with the sympathetic nervous system), nonchromaffin (associated with the parasympathetic nervous system), or mixed [4]. The concept was further queried on the basis of the classical endocrine role of the adrenal medulla and the chemoreceptor role of the nonchromaffin paraganglia. However, the linkage has now been confirmed by modern techniques with the demonstration of a common origin from neural crest and the production of a range of catecholamines. The relatively insensitive chromaffin reaction is no longer applicable in either diagnostic practice or research.

The adrenal glands were first described by the Italian anatomist Bartolomaeus Eustachius in the sixteenth century and the histology of the gland was described in the nineteenth century, when it was proposed that the medulla was

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related to the nervous system. Addison demonstrated the requirement for the adrenal cortex for life [5]. The role of the medulla was first demonstrated in 1894 [6] when adrenal extract injected into a dog was associated with a pressor response. The chemical responsible was isolated and named "epinephrin" [7] or "adrenalin" [8]. Norepinephrine (NE) (noradrenaline) was characterized later [9]. In the early 1950s, it was discovered that catecholamines were associated with intracytoplasmic granules in the adrenal medulla [10, 11] and this observation started the scientific investigation of the neurosecretory granule.

The human carotid body was described by Taube in 1743 and illustrated by Neubauer in 1772. Other historical aspects of paraganglia are extensively reviewed elsewhere [12].

12.3 Normal Structure and Function

12.3.1 Development

Paraganglia are derived from the neural crest. They are recognized in the human fetus by 7 weeks' gestation when they comprise small primitive cells. These give rise to neuroendocrine cells, neural cells, and glial (sustentacular) cells. In extra-adrenal paraganglia, differentiated cells replace primitive cells by week 25, but primitive cells persist for longer in the adrenal medulla. Here they originally lie in aggregates in the cortex, but then migrate to the area around the central vein. The medulla is recognizable by the sixth month of postnatal life. The largest aggregate in the fetus, and up to about 3 years, is the organ of Zuckerkandl, usually forming a fused horseshoe-shaped organ around the origin of the inferior mesenteric artery. This is thought to be the major source of catecholamines at that time, but atrophies and usually disintegrates into microscopic foci in the older child and adult [13].

12.3.2 Distribution of Paraganglia

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 organ(s) of Zuckerkandl. In the pelvis, they are in greatest numbers in association with the inferior hypogastric plexi entering the urogenital organs, in the bladder wall and in the sacral plexus [14]. Sympathetic paraganglia may also be found around the hilum of the kidney and in periadrenal fat, and sometimes in the thoracic region.

Parasympathetic paraganglia are more restricted in distribution, and are found 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 [15]. The carotid body constantly lies above the carotid bifurcation, but other parasympathetic paraganglia are variable in specific localization 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 [16]. Occasional reports have suggested that paraganglia may be found in other sites such as the gallbladder [17, 18], where they may be associated with tiny branches of the vagus nerve. Other locations, such as orbit and extremities, are more difficult to explain on embryological terms. As with sympathetic paraganglia, the amount of parasympathetic paraganglionic tissue at individual sites may change with age.

12.3.3 Function

All paraganglia can synthesize and secrete catecholamines, although the quantities produced by different paraganglia vary. Sympathetic paraganglia on average contain higher concentrations than parasympathetic. The biosynthetic pathway is shown in Fig. 12.1. Tyrosine comes from dietary sources or from the hepatic conversion of phenylalanine.

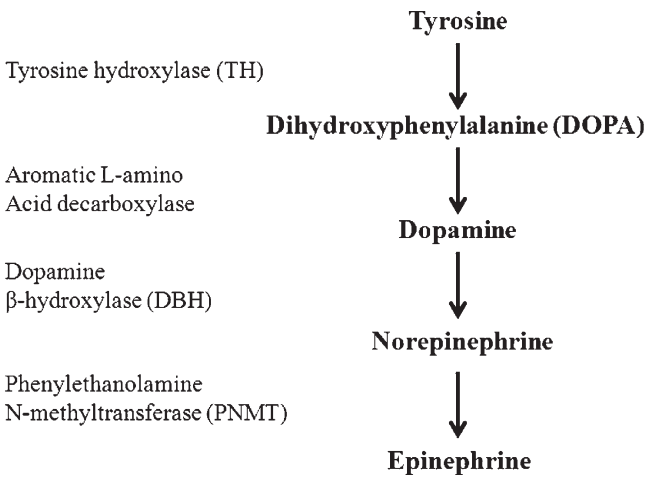

Fig. 12.1 Pathway of biosynthesis of catecholamines. The products and amounts of each vary with the paraganglion. For example, extra-adrenal sympathetic paraganglia produce mainly norepinephrine, whereas the adrenal produces mainly epinephrine

It is converted to dihydroxyphenylalanine by tyrosine hydroxylase 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 NE. NE diffuses back into the cytosol for conversion to epinephrine by phenylethanolamine *N*-methyltransferase (PNMT). This is transported back into secretory granules for storage or metabolized. The balance of the individual products varies with the particular paraganglion. NE predominates in the extra-adrenal sympathetic paraganglia. Epinephrine amounts to about 80% of catecholamines in the human gland. This is because glucocorticoids significantly increase the expression of the messenger RNA for PNMT and prevent the degradation of the enzyme [19]. Parasympathetic paraganglia produce very little NE or epinephrine, but may contain high levels of dopamine. In the adrenal medulla, secretion of catecholamines is largely regulated by preganglionic cholinergic nerves. The parasympathetic paraganglia function as chemoreceptors.

Catecholamines are stored in neurosecretory granules with a variety of proteins. Chromogranin A, an acidic glycoprotein, is a major component to which they bind. It regulates the formation of secretory granules and the release of hormones [20]. The other members of the family, chromogranin B and secretogranin I, are also stored but at much lower concentrations [21, 22]. A range of signaling peptides is also produced in paraganglia that may have paracrine roles. Neuropeptide Y and peptide YY are found in the adrenal medulla [23] as are galanin [24], adrenomedullin [25], vasoactive intestinal peptide (VIP), and pituitary adenylate cyclase-activating polypeptide [26]. Insulin-like growth factor 1, tumor necrosis factor-α, and interleukin-6 have also been reported [27] to be found. Basic fibroblast growth factors may have a number of roles [28]. They also seem to be expressed in the carotid body and play a significant role in function and growth [29].

12.3.4 Histology and Immunohistochemistry

In both types of paraganglia, the nests of neuroendocrine cells are surrounded by sustentacular cells, with a variable fibrovascular component. The pattern is more obvious in the 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 earlier. An alternative name in the adrenal medulla is pheochromocytes. In parasympathetic paraganglia, they are called type 1 or chief cells, or glomus cells. Hyaline eosinophilic globules, up to 20 μm in diameter, may be found in pheochromocytes of the adrenal medulla. These are periodic acid-Schiff positive and diastase resistant. Some cellular and nuclear pleomorphism may develop with increasing age. Ultrastructural analysis demonstrates the characteristic membrane-bound neurosecretory granules of varying shapes and sizes. The cells may also contain small synaptic-like vesicles that tend to sit close to the cell membrane [30]. Sustentacular cells (also known as satellite or type 2 cells) have a dendritic shape, but are not easily seen on hematoxylin and eosin staining. They may be localized by immunostaining for S100 protein [27, 31]. 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 [27, 32]. Ganglion cells and nerve fibers are commonly found in the adrenal medulla, where most of the neuroendocrine cells are innervated [33]. 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 general neuroendocrine markers including the neurosecretory granule protein chromogranin A [31, 34] and synaptophysin, a synaptic vesicle protein [35]. Neuron-specific enolase is not recommended as a marker 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 and SV2 [36, 37]. They will also stain positively for enzymes in the catecholamine biosynthetic pathway and for enzymes involved in processing peptide signaling molecules, including proconvertases [38] and peptidylglycine α-amidating monooxygenase [39].

Adrenal medullary nerves have been shown to be immunopositive for substance P [40] and pituitary adenylate cyclase-activating polypeptide [41]. The carotid body contains calcitonin gene-related peptide, substance P, galanin, VIP, and Neuropeptide Y immunoreactive fibers [42]. Nitric oxide may also to be important in the neural regulation of function of both types of paraganglia [42, 43].

12.4 Epidemiology of Paragangliomas

The true incidence of paragangliomas is not known, but an estimate of ~1/300,000 has been made [44]. An annual incidence of between 0.4 [45] and 9.5 [46] per 10^7 for pheochromocytoma has been reported and of 1 per 10^7 for paragangliomas of the head and neck [47]. On the basis of the relative distribution of sites [48], paragangliomas at other loci may have an incidence of 0.45 per 10^7 [44].

It is now known that at least 30% of paragangliomas arise on a background of genetic mutations [49, 50] and the distribution and behavior varies with the mutation. Approximately 10% of pheochromocytomas are associated with multiple endocrine neoplasia (MEN) types 2A and 2B, von Hippel–Lindau (VHL) syndrome, and neurofibromatosis type 1 (NF1), and these have been recognized for some time. About 50% of MEN2 patients develop pheochromocytoma [51] but extra-adrenal tumors are extremely rare. They coexist with medullary carcinoma of thyroid (MTC) and parathyroid hyperplasia in MEN2A and with MTC and mucocutaneous neuromas in MEN2B. They occur in 0.1–5.7% of patients with NF1 [52] and in 10–30% of those with VHL disease, in whom they characterize type 2 disease [1]. The major increase reflects mutations recently identified in the genes encoding the B, C, and D subunits of succinate dehydrogenase in about 20% of apparently sporadic paragangliomas, including extra-adrenal tumors [53]. These are now recognized as the familial paraganglioma syndromes (PGL1, 3, and 4). The distribution of the lesions varies with the gene involved (Table 12.1) [50]. There may also be other, as yet unidentified, susceptibility genes [54].

These inherited tumors appear to segregate into two groups on the basis of gene expression profiling; those with *VHL*, *SDHB*, and *SDHD* mutations showing high expression of hypoxia, angiogenesis, and matrix-related genes, whereas those with *RET* and *NF1* mutations have changes consistent with activation of the Ras MAPK pathway [55]. The genes involved in familial disease do not appear to play significant roles in the pathogenesis of sporadic tumors, with *RET* mutations in up to 10% and *VHL* mutations in about 4% [56]. However, since the patterns of gene expression are similar in inherited and sporadic disease, it suggests that other genes encoding proteins in the same pathways may be involved.

Other genetic changes have been described and some segregate with syndromes. A deletion in 1p is the most common, occurring in about 80% of sporadic and MEN2 cases, but in only 15% of VHL tumors [57]. Other loci frequently involved are 3p, 3q, 11q, 17p, and 22q [57–61].

Table 12.1 Distribution of paragangliomas in familial disease [50]

Syndrome	Gene	Adrenal	Extra-adrenal sympathetic	Parasympathetic
MEN 2A and 2B	*RET*	++	±	–
VHL	*VHL*	++	++	±
NF1	*NF1*	++	±	±
PGL1	*SDHD*	+	++	++
PGL3	*SDHC*	±	±	++
PGL4	*SDHB*	+	++	++

MEN multiple endocrine neoplasia, VHL von Hippel–Lindau, NF1 neurofibromatosis type 1, PGL paraganglioma, SDH succinic dehydrogenase, subunits B, C, or D
++ usual site, + less common, ± rare

Paragangliomas of both types may be found in association with pulmonary chondroma and gastrointestinal stromal tumor in Carney's triad [62]. An autosomal dominant syndrome has also been described in which pheochromocytoma coexists with gastric stromal sarcoma [63].

12.5 Sympathetic Paragangliomas

12.5.1 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. In adults, more than 90% of tumors are intra-adrenal, but in children, they are more commonly extra-adrenal tumors [64]. About half of extra-adrenal tumors arise in the organs of Zuckerkandl and the rest appear mainly in the retroperitoneum. Other sites include the bladder [49], with occasional lesions reported in the kidney, urethra, prostate, and gallbladder [65]. Less than 2% occur in thoracic or cervical locations [66]. In general, there is an almost equal sex distribution, but males are reported to be more commonly affected among children and patients with thoracic tumors [67].

12.5.2 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 [68] and 6 g at hospital autopsy, reflecting the hypertrophy of the cortex associated with stimulation by adrenocorticotrophin in the stress of terminal illness [69]. The medulla accounts for about 10% of the normal gland [68, 70] 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 [71]. There is irregularity of the cortical–medullary junction in the human adrenal gland and intermingling of cortical and medullary cells [72], consistent with the fact that each influences the function of the other [73].

12.5.3 Pheochromocytoma

It is now recognized that up to 50% of pheochromocytomas may be associated with familial syndromes, as outlined earlier. Sporadic tumors are usually solitary in contrast to familial disease where more than 50% are bilateral. In some of the familial

syndromes, they may coexist with extra-adrenal sympathetic and/or parasympathetic paragangliomas (Table 12.1). Thus, the finding of more than one paraganglioma in any individual indicates the need for a detailed family history, and probably appropriate genetic testing. In children, multifocal and extra-adrenal paragangliomas have been reported in 30–43% of cases [74]. This may reflect a higher incidence of familial disease. 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 does not give rise to such symptoms, possibly because enzymes of the catecholamine biosynthetic pathway are not expressed. Alternatively, this may be due to metabolism of catecholamines to inactive metabolites by the tumor cells.

In the past, a large number of pheochromocytomas were first diagnosed only at autopsy [75, 76]. Some still have a primary diagnosis after death, and they may account for 0.05% of coroners' autopsies [77]. Diagnosis may be more difficult in the elderly [78]. Undiagnosed lesions now also account for a significant number of adrenal "incidentalomas," picked up when the abdomen is scanned for the investigation of other intra-abdominal disease [79–82].

The lesions are usually intra-adrenal (Fig. 12.2) and may appear encapsulated. The normal gland can be easily identified in many cases but may be attenuated over the surface, particularly in large tumors. Most are between 3 and 5 cm in diameter [83] but the size may range from 1 to greater than 10 cm. The weight ranges from less than 5 to over 3,500 g, the average weight in patients with hypertension being 100 g [84]. 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. 12.3). Some tumors show calcification. There may be evidence of direct invasion of surrounding structures, such as kidney or liver. Rarely, there is extension into the inferior vena cava. However, although this pattern of growth may be lethal, it does not correlate with metastatic potential and is no longer regarded as malignant behavior. In some cases, there is evidence of distant metastasis at the time of presentation.

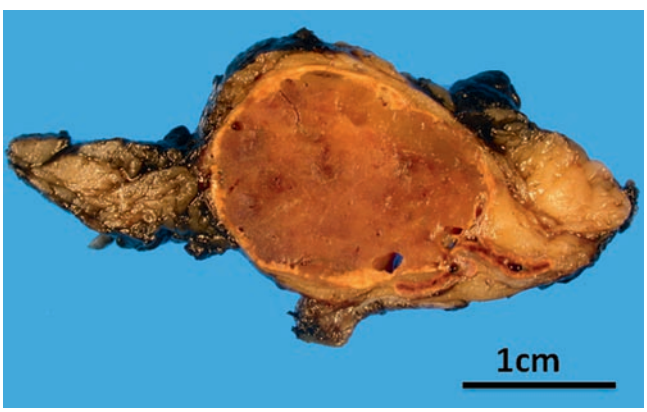

Fig. 12.2 Pheochromocytoma. The tumor is intra-adrenal and the paratumoral gland can be seen around the edge and to the bottom right. There is no evidence of medullary hyperplasia

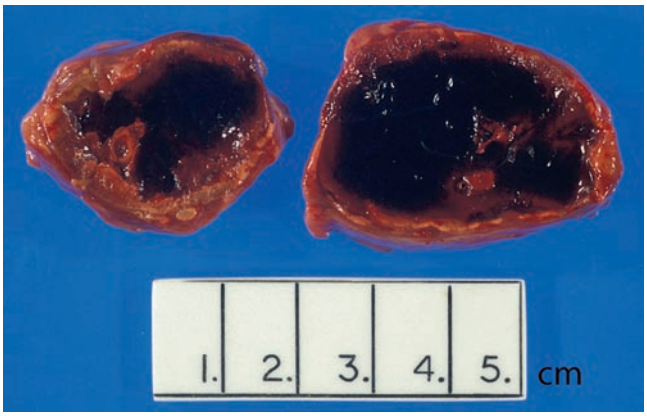

Fig. 12.3 Pheochromocytoma showing hemorrhage and cystic degeneration. The adrenal cortex can be seen attenuated over the surface

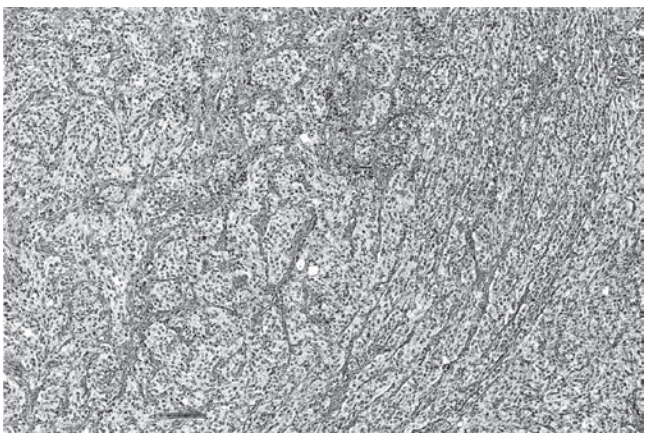

Fig. 12.4 Pheochromocytoma showing mixed alveolar (*left*) and trabecular (*right*) architecture. Hematoxylin and eosin

Microscopic examination most commonly shows a mixed alveolar and trabecular arrangement (Fig. 12.4). In some tumors, one or other of these patterns predominates (Fig. 12.5). The cell nests may vary in size (Fig. 12.6) and some have suggested that large cell nests are more commonly seen in malignant tumors [85, 86]. In about 2% of cases, a spindle component may be found, but only rarely predominates. 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

Fig. 12.5 Pheochromocytoma showing alveolar pattern. Hematoxylin and eosin

(Fig. 12.7) is pronounced and nuclear pseudoinclusions are seen [87], whereas others are composed of small cells [88]. Intracellular hyaline globules may be a feature (Fig. 12.8). Variable amounts of melanin-like pigment, neuromelanin, derived from a combination of breakdown products of tyrosine and lipofuscin may be seen occasionally (Fig. 12.9) [89, 90]. Neuromelanin 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 1/30 high power fields reported in clinically benign lesions in one study [91]. Cells resembling ganglion cells and neuroblasts are occasionally seen. Sometimes the tumor cells undergo "lipid degeneration," assuming a clear cell appearance, which may mimic an adrenal cortical tumor (Fig. 12.10) [92, 93]. Oncocytic tumors have been described [94]. Stromal sclerosis may be marked and amyloid has been demonstrated [95, 96]. The vascular component is often prominent.

There are few data on genotype–phenotype correlations in syndromic pheochromocytomas. However, it has been reported that VHL tumors have a thick vascular capsule, hyaline and myxoid stroma, absence of intracytoplasmic globules, and lack of nuclear atypia and mitoses [97].

The basis of specific diagnosis is immunohistochemistry, and nonspecific histochemical techniques such as the chromaffin reaction and silver stains are no longer relevant. As in other neuroendocrine tumors, there is immunopositivity for the general neuroendocrine markers synaptophysin and chromogranin A (Fig. 12.11). They may also express neurofilament [98]. Immunostaining for S100 protein will demonstrate sustentacular cells (Fig. 12.12) [99, 100], 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 glial fibrillary acidic protein. A number of the peptides produced by the

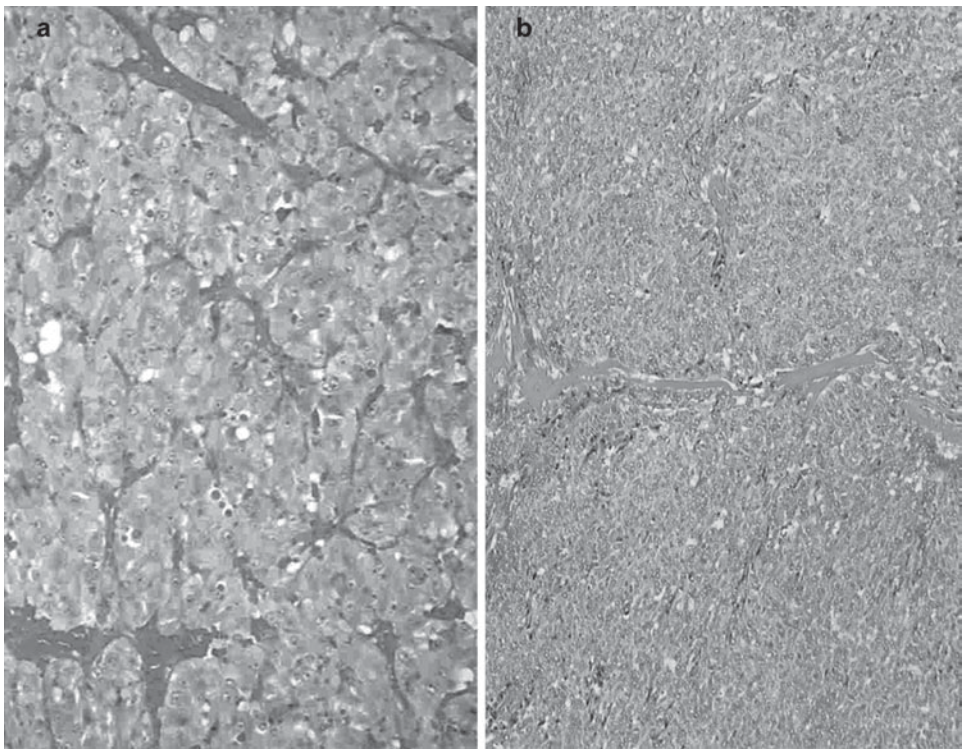

Fig. 12.6 (**a**) This pheochromocytoma comprises cells arranged in small nests, hematoxylin and eosin. (**b**) In this tumor, there are mainly large nests. This pattern is reported to be more common in malignant tumors. Hematoxylin and eosin

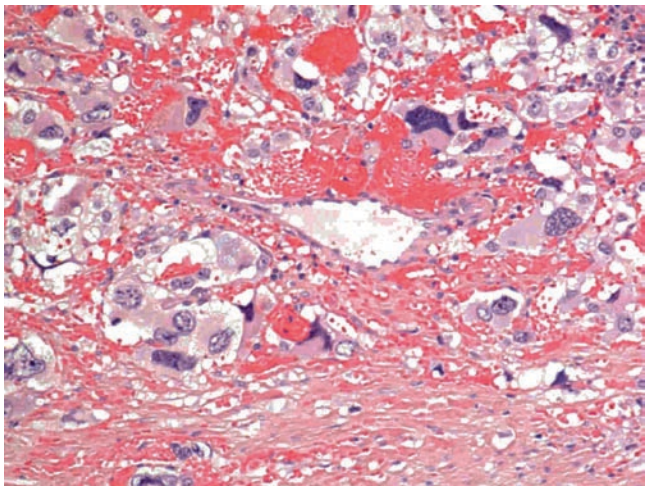

Fig. 12.7 Pheochromocytoma showing marked nuclear pleomorphism. Hematoxylin and eosin

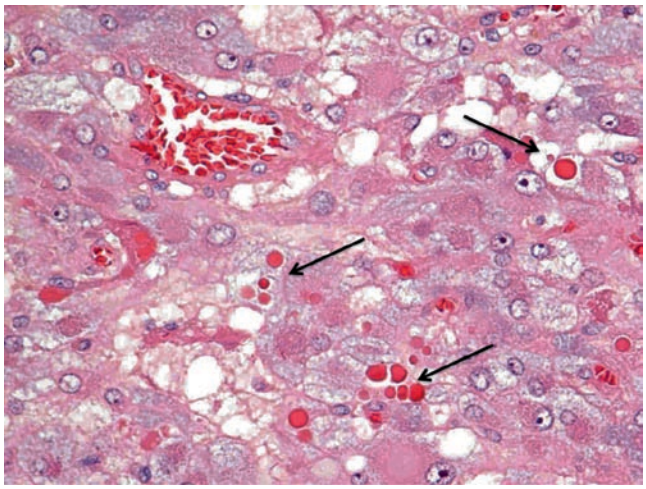

Fig. 12.8 Pheochromocytoma showing intracellular hyaline globules (*arrows*). These contrast with the red blood cells seen in the upper left of the figure. Hematoxylin and eosin

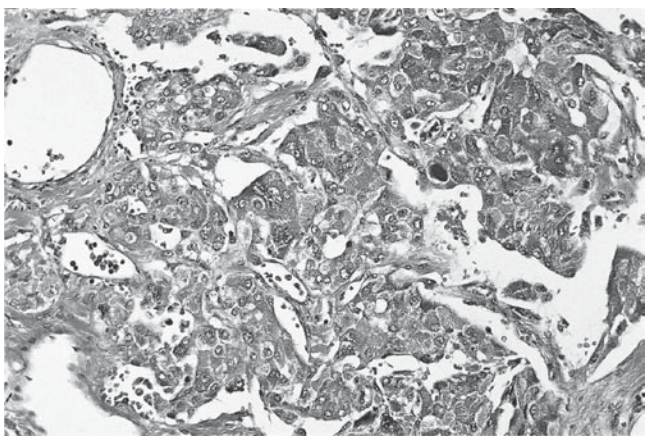

Fig. 12.9 In this pheochromocytoma, there was a significant amount of brown pigment in the tumor cells. It gave positive staining for melanin. Hematoxylin and eosin

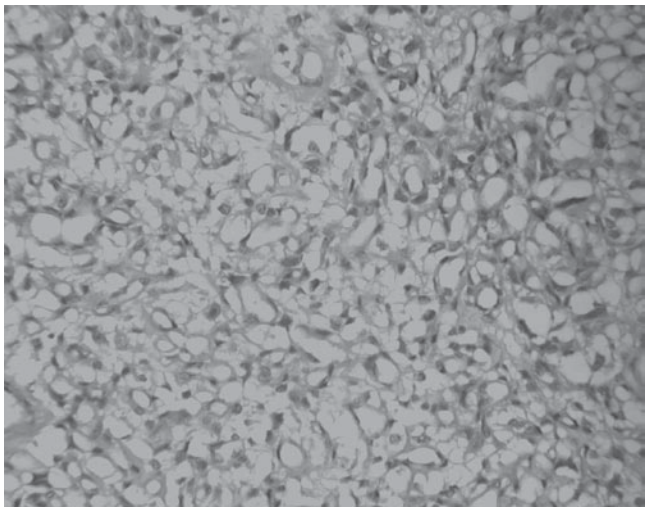

Fig. 12.10 Pheochromocytoma showing clearing of tumor cells, mimicking an adrenal cortical tumor. Hematoxylin and eosin

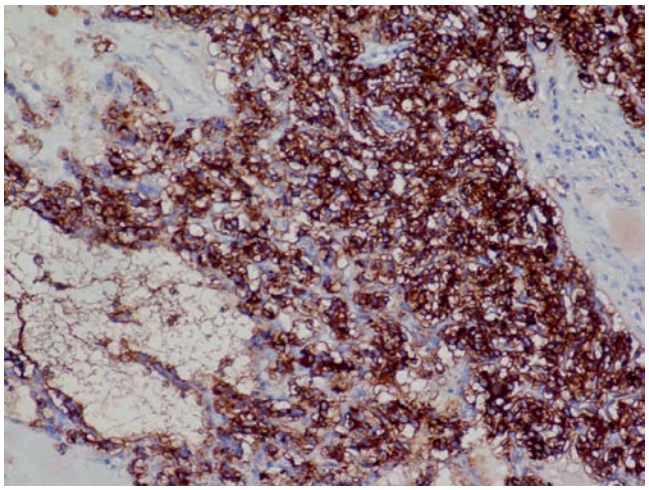

Fig. 12.11 Pheochromocytoma showing strong positivity for chromogranin A, a general neuroendocrine marker. Immunoperoxidase

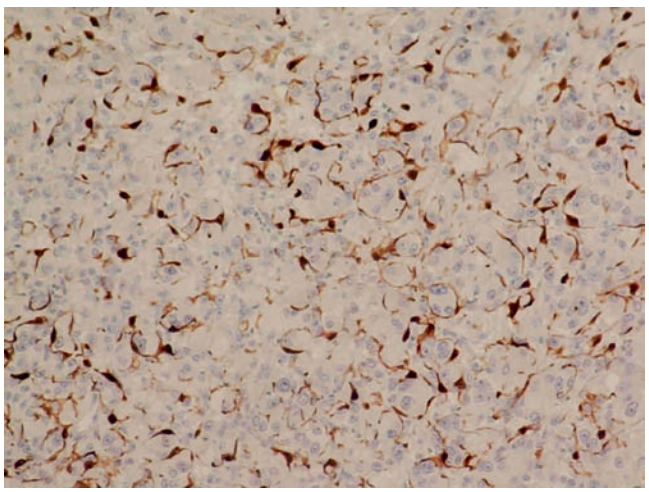

Fig. 12.12 Pheochromocytoma showing large numbers of sutentacular cells, immunopositive for S100 protein. The cell processes extend around small groups of tumor cells. Immunoperoxidase

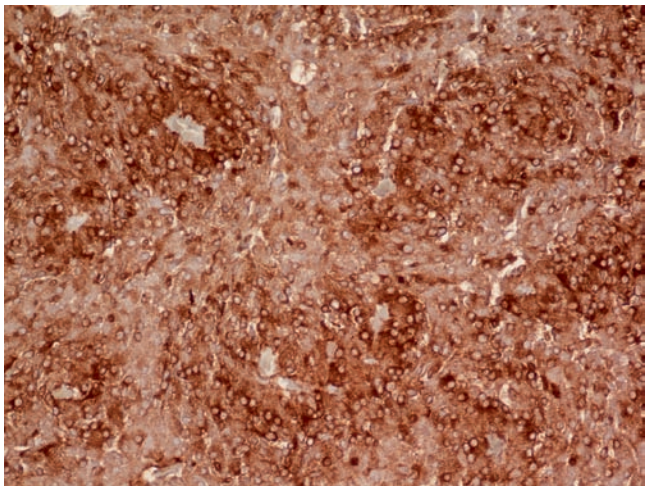

Fig. 12.13 Pheochromocytoma showing heterogenous positive staining for tyrosine hydroxylase. Immunoperoxidase

normal medulla are also expressed in tumors [101–103], although these are not normally documented as a diagnostic procedure. The exception would be the demonstration of hormones such as adrenocorticotrophin in cases of ectopic hormone secretion associated with a clinical syndrome [104, 105]. Specific identification of paraganglioma can be made by immunopositivity for the enzymes involved in the synthesis of catecholamines such as tyrosine hydroxylase (Fig. 12.13) and negative staining for markers of other types of neuroendocrine tumor [50, 106, 107].

12.5.4 Extra-Adrenal Sympathetic Paragangliomas

The relative distribution of extra-adrenal paragangliomas is shown in Table 12.2. Between 25% and 86% of intra-abdominal tumors are functional and are almost always noradrenergic because they do not express PNMT, the enzyme that converts NE to epinephrine (Fig. 12.1) [35]. Up to 50% of cases give rise to metastases [108, 109]. Histologically, they comprise nests of tumor cells surrounded by sustentacular cells producing the characteristic "zellballen" appearance.

Urinary bladder tumors are worthy of mention. They affect males and females equally, but may affect women at an earlier age than men [110]. They usually occur in the trigone, but may be found in the dome or lateral walls. The majority of patients have the clinical triad of paroxysmal hypertension, gross intermittent hematuria, and intermittent 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 and may project into the bladder lumen. Many

Table 12.2 Distribution of extra-adrenal sympathetic paragangliomas [139]

Site	Percentage of cases
Head and neck	3
Thorax	12
Intra-abdominal	85
Superior para-aortic	46
Inferior para-aortic	29
Bladder	10

interdigitate with muscle bundles, but this does not necessarily indicate malignant potential.

Intrathoracic paravertebral paragangliomas lie close to the sympathetic axis and are most commonly found in the midthoracic region [67]. 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 [111]. These tumors are consistently immunopositive for cytokeratin, and occasional lesions at other sites may show positivity [112–115].

12.5.5 Composite Pheochromocytoma

Because the embryologic precursor cells of paraganglia have the potential to give rise to neuroendocrine cells, nerves, and ganglion cells, pheochromocytomas may contain a few cells resembling ganglion cells or neuroblasts. Sometimes this is so prominent as to warrant a diagnosis of composite pheochromocytoma [116, 117], constituting 3% of cases in one series [91]. The second component may resemble neuroblastoma, ganglioneuroblastoma, ganglioneuroma, or, rarely, malignant peripheral nerve sheath tumor [118–120]. The behavior of such tumors is similar to pheochromocytoma when the second component is benign [91]. However, it is not possible to predict when it is histologically malignant. Some have shown no evidence of recurrence after 5 years [121]. Where metastases occur, they are usually to liver, lung, lymph nodes, and bone [1].

12.6 Parasympathetic Paragangliomas

Carotid body and jugulotympanic tumors are more common than vagal and aortic lesions [1]. They present usually in the fourth or fifth decades with an almost equal sex distribution [12, 122]. Only about 1% produces symptoms of catecholamine excess [123], which may reflect the fact that physiologically they produce only small quantities of catecholamines.

Carotid body tumors are bilateral in 3–8% of sporadic cases and 38% of familial cases [123, 124]. The majority show some adherence to the adventitia of the carotid artery and some completely surround the bifurcation [125]. A chronic inflammatory infiltrate is not uncommon. The incidence of paraganglioma of the carotid body is ten times greater in people living at high altitude than at sea level, and in this group, they are much more common in women [126, 127]. This may be related to the hyperplasia induced at altitude [128] in response to the hypoxic stimulus. Hypertrophy and hyperplasia also occur in people with chronic obstructive airways disease [129–132] and in patients with cystic fibrosis and cyanotic heart disease [133]. Increased proliferation has been demonstrated in the carotid bodies of rats subjected to hypoxia [134].

Jugulotympanic paragangliomas are slow-growing lesions that usually present in the fifth and sixth decades, with a female to male ratio of 6:1 [135]. Glomus jugulare tumors produce cranial nerve palsies, while glomus tympanicum tumors produce tinnitus and hearing loss. If they grow intracranially, they may mimic meningioma. 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 commoner in females. 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) [136, 137] or pericardium [138]. Pulmonary lesions may lie within the lung or near the pulmonary artery [139]. 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.

12.7 Diagnosis of Malignancy

On the basis of the 2004 classification of endocrine tumors, malignancy is now diagnosed only when there is metastasis to sites where paraganglial tissue is not normally found [1]. This helps avoid interpreting multiple primary lesions as metastatic. The rate of malignancy varies with the site. Only about 5% of pheochromocytomas metastasize, but up to 10% show local recurrence. However, 30% of extra-adrenal intra-abdominal tumors are malignant, while 5% [140] to 13.8% [141] of bladder lesions have metastasized to local lymph nodes or distant sites. The incidence of malignancy varies significantly with the particular inherited mutation in familial disease. Malignancy is very rare in NF1- and RET-associated

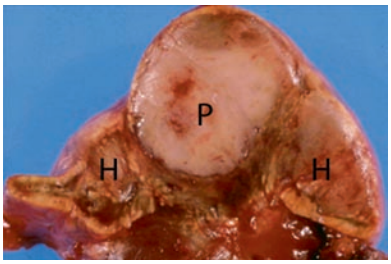

Fig. 12.14 Adrenal gland from a patient with MEN 2A. It shows a central pheochromocytoma (*P*). There is also adrenal medullary hyperplasia (*H*), with medullary tissue present in large amounts in the alae. Hyperplasia is a well-recognized precursor to pheochromocytoma in this syndrome

lesions but over 50% of paragangliomas associated with *SDHB* mutations are malignant [142].

There are no absolute histological criteria for predicting malignant potential, and therefore none of these lesions can at present be unequivocally defined as benign. Published data on a large series of sympathoadrenal paragangliomas found only four features that had a statistical correlation with malignancy, extra-adrenal location, coarse nodularity of the primary tumor, confluent tumor necrosis, and absence of hyaline globules [91]. Malignant tumors are heavier than benign tumors (average of 383 g compared with 73 g), have a higher mitotic count (average of 3 compared with 1 per 30 high power fields), and have extensive local or vascular invasion. Small cell morphology is more commonly seen in malignant tumors [88]. There is a correlation between depletion of sustentacular cells and malignant behavior but this is not absolute [143, 144].

A number of groups have attempted to correlate Ki-67 proliferation index with behavior (Fig. 12.14). However, methods have differed and it is difficult to compare findings. While there is a trend toward higher indices in malignant tumors, some have low levels of proliferation [145–147], and using 2–3% as the threshold, there was only 50% sensitivity in detecting malignant tumors. Ploidy analysis is not of diagnostic value [148, 149].

There have been two recent publications proposing multifactorial approaches to the diagnosis in sympathetic paragangliomas. They have not been tested on parasympathetic tumors. Both attribute a weighted score to a number of features and the total indicates the probability of benign or malignant behavior. The first is based on hematoxylin and eosin histology and was applied only to pheochromocytoma (Pheochromocytoma of the Adrenal gland Scaled Score, PASS) [86]. The details are shown in Table 12.3. In the initial series, all tumors that had metastasized had a score of ≥4. This has recently been validated in an independent group [145], in which all metastasizing tumors had an index of ≥6. The authors proposed that patients with a PASS score of ≥4 should be followed up closely because of the risk of recurrence.

Table 12.3 Proposed pheochromocytoma of the adrenal gland scoring scale (PASS score) [86]

Feature	Score
Large nests of cells or diffuse growth >10% of tumor volume	2
Necrosis (confluent or central in large cell nests)	2
High cellularity	2
Cellular monotony	2
Presence of spindle-shaped tumor cells (even focal)	2
Mitotic figures (>3/10 high power fields)	2
Extension of tumor into adjacent fat	2
Vascular invasion	1
Capsular invasion	1
Profound nuclear pleomorphism	1
Nuclear hyperchromasia	1
Total possible score	20

All tumors that metastasized were reported to have scores ≥4

Table 12.4 Proposed scoring scale for pheochromocytomas and extra-adrenal sympathetic paragangliomas [85]

Feature	Score
Histological pattern	0
Uniform cell nests	1
Large and irregular cell nests	1
Pseudorosettes (even focal)	
Cellularity	0
Low (<150 cells/mm^2)	1
Moderate (150–250 cells/mm^2)	2
High (more than 250 cells/mm^2)	
Necrosis (confluent or central in large cell nests)	2
Vascular or capsular invasion	1
Ki-67 index	0
<1% or 20 cells per medium power field (×200)[a]	1
>1% or 20 cells per medium power field[a]	2
>3% or 50 cells per medium power field[a]	
Catecholamine phenotype	0
Adrenergic	1
Noradrenergic or "nonfunctional"	
Total possible score	10

[a]Average count of 20 fields in area of highest labeling

In the second model, both pheochromocytomas and extra-adrenal sympathetic paragangliomas were included. Histologic features were combined with proliferative activity, as assessed by Ki-67 labeling index, and with the catecholamine phenotype of the tumor (Table 12.4) on the basis of which the tumors were classified as well, moderately, or poorly differentiated [85]. This approach did not give 100% discrimination but the proportion of malignant tumors increased with the decrease in differentiation (Table 12.5). While these models require further validation, they may prove useful in identifying patients with a higher risk.

A number of other markers have been reported to correlate with behavior, but none has yet found a way into diagnostic practice. The expression of CD-44S was associated with lack of metastases in patients with a PASS score ≥4 [150]. Heat-shock protein 90, N-cadherin, vascular endothelial growth factor [151], and breakdown products of chromogranins B and C [34] have helped distinguish malignant from benign. Expression of telomerase is also more common in malignant lesions [152–154]. Immunopositivity for p53, Bcl-2, mdm-2, cyclin D1, p21, and p27 did not discriminate [145].

Table 12.5 Outcome related to score [85]

Score	Metastases (%)	5-year survival (%)
1–2	13	92
3–6	63	69
7–10	100	0

Interesting data are emerging from the molecular genetic analysis of these lesions and these may in future help with diagnosis and prognosis. Malignant tumors have more copy number changes than benign as identified by comparative genomic hybridization [150]. Expression profiling has identified gene sets that appear to segregate the two groups [155, 156].

12.8 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 an intra-abdominal 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 [157], and occasional expression of cytokeratins is seen in paragangliomas [158].

In the neck, they may need to be distinguished from MTC. This can usually be done on the basis of widespread positivity for calcitonin and carcinoembryonic antigen [159] and thyroid transcription factor 1 [160] in medullary carcinoma. Sustentacular-like cells have been reported in medullary carcinoma [161, 162], so S100 staining is not useful. Hyalinizing trabecular tumors of thyroid are characterized by immunopositivity for cytokeratin [163, 164], so negative staining can rule out this as a differential diagnosis. However, as some paragangliomas in this region express cytokeratin [115, 158], positive staining is unhelpful. 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 carcinoembryonic antigen [165].

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. 12.10) [92, 93]. Synaptophysin and PGP 9.5 can be expressed by adrenal tumors [166, 167], so chromogranin A is the only useful neuroendocrine marker in making the distinction. A combination of inhibin-α, calretinin, and Melan-A (Clone A103) will identify the majority of adrenal cortical tumors [168–171].

Pigmented paragangliomas may need to be distinguished from metastatic melanoma. Melan-A (either antibody) should identify melanomas [172], and is negative in paragangliomas [168]. HMB45 may occasionally stain pheochromocytoma [173, 174]. Rarely, melanoma may be a primary at this site [175, 176].

12.9 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 [71, 177], but in general, diagnostic practice is recognized as 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 [178]. 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 [71]. 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 [179]. The demonstration of clonal lesions in both of these groups suggests that some lesions less than 1 cm may indeed be neoplastic rather than hyperplastic [180]. AMH is a well-recognized precursor to pheochromocytoma in MEN 2 [71, 179], but does not seem to be involved in those associated with VHL [97].

AMH has also been described occasionally in sudden infant death syndrome [181], in Beckwith–Wiedemann syndrome, and in association with adrenocortical adenoma [182, 183]. These may represent chance associations rather than causal events.

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Chapter 13
Molecular Biology of Pheochromocytomas and Paragangliomas

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13.1 Introduction

Pheochromocytoma is a tumor arising from the chromaffin cells of the adrenal medulla, and its counterpart, paraganglioma, arises from sympathetic ganglia outside the adrenals [1]. Most pheochromocytomas and paragangliomas develop sporadically, but a significant number of cases, approximately 30%, are inherited as part of a familial syndrome (Fig. 13.1). These syndromes include multiple endocrine neoplasia type 2, von Hippel Lindau disease, neurofibromatosis type 1, and the familial paraganglioma/pheochromocytoma syndrome, characterized by mutations in the *RET*, *VHL*, *NF1*, and *SDH* genes, respectively. More recently, novel susceptibility genes associated with familial forms of pheochromocytoma have been identified. The intriguing molecular pathways underlying these conditions are being exposed in increasing detail, and it is now hypothesized that these genes interact on various levels and might share a defective apoptotic pathway of sympathetic lineage precursor cells during embryogenesis. As more is understood regarding the complex molecular biology underlying these rare tumors, greater insight into patient screening and therapeutic options may be attained and perhaps applied to other types of neoplasia.

13.2 Multiple Endocrine Neoplasia

Multiple endocrine neoplasia (MEN) describes an array of inherited endocrine neoplasias and classically is divided into types 1 (MEN1) and 2 (MEN2) [2, 3]. MEN2 is further classified into groups 2A and 2B depending on the clinical features present in the disease. MEN2A is characterized by medullary thyroid carcinoma (MTC), and is often accompanied by pheochromocytoma and hyperparathyroidism. MEN2B also includes MTC and pheochromocytoma, along with a myriad of additional findings including ganglioneuromatosis, multiple neuromas, marfanoid body habitus, and thickened corneal fibers [2, 3]. In contrast, a distinct set of clinical features comprises MEN1. Pheochromocytoma is not usually involved in MEN1 and thus this syndrome is outside the scope of this chapter.

MEN2, inherited in an autosomal dominant pattern, results from germline mutations of the *RET* gene [3]. This particular mutation is unique amongst those associated with most familial neoplasms, as it is an activating mutation of an oncogene. This is in contrast to the usual pattern of inactivation of a tumor-suppressor gene seen in most inherited cancer syndromes as we will discuss later [3]. The *RET* gene, under normal circumstances, encodes a transmembrane receptor tyrosine kinase that is necessary for normal growth and development of neural crest derived cells [3]. In order to signal downstream events, this receptor interacts with two proteins: the soluble glial cell line-derived neurotrophic factor (GDNF), which functions as a co-receptor, and the GDNF receptor alpha extracellular protein. Once GDNF binds to GDNFα, the resulting complex binds to the extracellular domain of the RET tyrosine kinase receptor and triggers dimerization and autophosphorylation [3]. This autophosphorylation signals a cascade of intracellular events.

In MEN2A, missense mutations in the extracellular domain of *RET*, most commonly found in exons 10 and 11, interrupt the normal sequence of codons in its cysteine-rich domain such that a cysteine is left unpaired [4]. This allows formation of an alternative intra- or intermolecular disulfide bond that may contribute to ligand-independent homodimerization and inappropriate activation of the RET protein [3, 5]. Upon activation, autophosphorylation of multiple intracellular tyrosine residues ensues, triggering downstream signaling pathways. Of particular importance is tyrosine residue 1,062 which serves as a binding site for multiple adaptor proteins. This residue and its adaptors function to activate the RAS/ERK, PI3K/AKT, p38MAPK, and JNK pathways, stimulating growth, differentiation, and cellular survival mechanisms [3, 6].

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In MEN2B, mutations affect the catalytic site of the kinase, leading to impaired recognition of its substrates and a resultant alteration of the ideal binding site [3]. As seen in MEN2A, an altered autophosphorylation pattern leads to abnormal downstream signaling. However, in MEN2B, the mutation causes loss of phosphorylation at some sites but also produces a novel autophosphorylation site which alters substrate specificity [4, 7, 8]. Mutations of exon 16 and rarely in exon 15 are the genetic hallmarks of MEN2B [3].

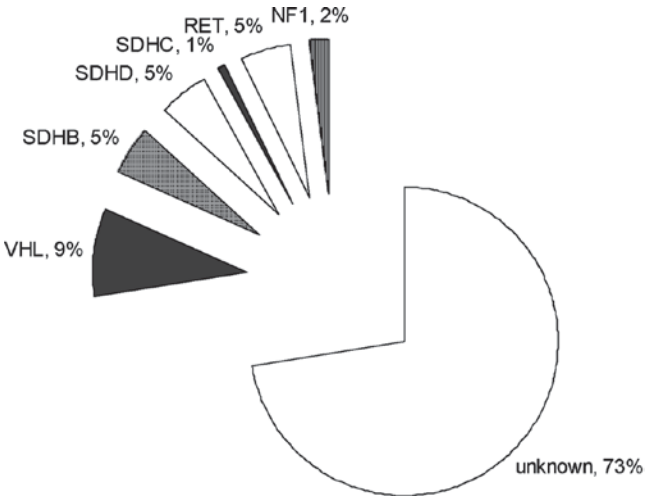

Fig. 13.1 Frequency of detectable mutations in pheochromocytoma susceptibility genes based on results from various series (numbers shown are approximate, please refer to text for additional details). The majority of pheochromocytomas and paragangliomas, including some familial forms and sporadic-appearing tumors, still remain without an identifiable genetic defect

Genotype-phenotype correlations in MEN disease are directly implicated in screening and treatment of affected patients and their relatives (Table 13.1) [9–11]. The first clinical manifestation of disease is most often MTC, and any patient diagnosed with apparently sporadic MTC should undergo *RET* testing [2, 12, 13]. Children of families identified as carriers of an MEN2-specific *RET* mutation should be offered "prophylactic thyroidectomy" as early as 4–6 years of age [2]. The exact timing of thyroidectomy can be guided by the patient's *RET* mutation, as genotype-phenotype correlations are predictive of disease onset and MTC aggressiveness in many instances [14]. Three categories of risk (levels 1–3) have been outlined in the literature to predict MTC aggressiveness, in conjunction with the mutated codon [13, 15–17]. In cases of MEN2B, it is commonly argued that prophylactic thyroidectomy be performed within the first year of life [18]. MEN families, as they are at increased risk for development of pheochromocytoma, should undergo biochemical testing annually with plasma or urinary catecholamines and/or metanephrine measurement. Metanephrines, either plasma-free or urinary-fractionated, are generally considered to be the most sensitive and specific of available

Table 13.1 Genotype–phenotype correlations of RET mutations

Phenotype	Codon	Exon	Additional clinical features	Disease aggressiveness[a]	Early or late onset
MEN 2B	918[b]	16	MTC, ganglioneuromatosis, multiple neuromas, marfanoid body habitus, and thickened corneal fibers	+++	Earliest
	883	15	Unknown PC frequency(rare)	+++	Earliest
MEN 2A only	635		MTC, Parathyroid hyperplasia	Unknown	Unknown
	637			Unknown	Unknown
MEN 2A	609	10	Hirschsprung, PC rare	+	Late
	611	10	No PC identified	++	
Or	618	10	PC in 14%	++	Late
	620	10	Hirschsprung, PC rare	++	Late
FMTC	634[b]	11	Rare CLA, bilateral PC in up to 50%	++	Early or late
	790	13	Rare PC	+	Late
	791	13	Rare PC	+	Late
	804	14	Rare PC	+	Early or late
	891	15	Rare PC	+	Late
FMTC only	532	8		Unknown	Unknown
	533	8		Unknown	Unknown
	630	11		++	
	768	13		+	
	844	14		+	
	912	16		+	

[a]Mainly based on mMTC phenotype[b]Indicates most common mutation of the phenotype

MEN2A or 2B = multiple endocrine neoplasia type 2A or 2B, *CLA* cutaneous lichen amyloidosis, *PC* pheochromocytoma, *MTC* medullary thyroid carcinoma, *FMTC* familial medullary thyroid carcinoma

assays [2, 19]. On the basis of multiple studies designed to analyze the diagnostic accuracy of plasma-free metanephrines, the combined sensitivity and specificity were calculated to be 98% and 92%, respectively. Metanephrines provide a more reliable measurement of tumor activity, as they are produced in large amounts and stored in the chromaffin granules. On the other hand, catecholamines may be released intermittently or in small amounts, making their measurement unreliable [19]. Multiple-imaging modalities, including computed tomography, magnetic resonance imaging, iodine-131 metaiodobenzylguanidine scanning, and positron emission tomography may be utilized to visualize pheochromocytoma. However, with only rare exceptions, the current recommendation is to employ imaging only when biochemical testing indicates disease [19, 20].

While most of the known mutations implicated in MEN2A are highly penetrant and warrant early and aggressive screening and treatment as mentioned above, mutations of codon 804 in exon 14 seem to be more variable resulting in an unpredictable phenotype. This particular mutation was initially thought to be associated only with familial medullary thyroid cancer but was extended to the MEN2A phenotype when the mutation was also identified in patients with pheochromocytoma [21]. As seen in the initial case report of MEN2A associated with a codon 804 mutation, patients may present relatively late in life with mild disease (Table 13.1). However, there have also been case reports of aggressive phenotypes associated with this mutation [13], leading to the recommendation that the usual screening practices be maintained in patients harboring the 804 mutation [13, 21–25].

Surgical resection remains the mainstay of treatment for pheochromocytoma, regardless of whether the disease is sporadic or familial. In cases of benign familial diseases such as MEN, the risk of recurrence is significant, and pheochromocytoma is more likely to involve both adrenals. There are now data to support the practice of performing cortical-sparing adrenalectomy with the goal of minimizing the morbidity associated with bilateral adrenalectomy and resultant insufficiency. Approximately 15–30% of adrenal tissue must be preserved in order to avoid lifelong glucocorticoid and mineralocorticoid replacement [16, 26].

13.3 von Hippel-Lindau Disease

von Hippel-Lindau disease (VHL) refers to a condition in which individuals affected by the disease carry an increased risk of developing CNS hemangioblastomas, renal cysts and renal cell carcinoma, and pheochromocytoma [27]. Additional tumors may also be seen including pancreatic islet cell tumors, endolymphatic sac tumors, and broad ligament cystadenomas [27].

The *VHL* gene, characterized as a tumor suppressor gene, is located on chromosome 3p25, and VHL disease is inherited in an autosomal dominant fashion [28]. The gene has widespread expression, but VHL disease manifests itself in only several distinct tissues. While this phenomenon is not readily explained, there are clear genotype–phenotype correlations which allow for subclassification of disease [29]. Type 1 VHL disease confers a low risk for pheochromocytoma, whereas type 2 disease is seen in families with increased risk of pheochromocytoma. Type 2 disease can be further separated into 3 categories, types 2A, 2B, and 2C, featuring increased risk of renal cell carcinoma, decreased risk of renal cell carcinoma, and pheochromocytoma only, respectively. Missense mutations are seen in all groups of type 2 disease, while type 1 disease may have deletion, missense, or nonsense mutations of the *VHL* gene. Another distinction between the 2 groups involves the VHL protein (pVHL) and its function, as discussed below [27].

The *VHL* gene may encode either a long or short form of its protein depending on the start codon used by the gene, but despite the presence of these two isoforms, VHL disease mutations are found in a region shared by both. The components of the protein include both alpha and beta subdomains. The alpha domain is involved in forming a multiprotein complex, which functions in proteosomal degradation. The beta domain acts as a substrate docking site [27]. One of the most significant substrates that interacts with pVHL is the transcription factor, hypoxia inducible factor (HIF), and its three alpha subunits, HIF1α, HIF2α and HIF3α [27]. HIF represents the best known cellular response to hypoxic conditions by activating genes responsible for mechanisms such as glucose uptake and metabolism, angiogenesis, and erythropoiesis [27]. Under normal conditions, pVHL interacts with and targets HIFα for degradation once it is hydroxylated at two specific prolyl residues by a member of the prolyl hydroxylase family [27, 30]. In VHL-related pheochromocytoma, HIF activity is enhanced, as pVHL cannot target HIF for degradation when mutated (Fig. 13.2). One exception to this, however, is seen in VHL type 2C disease, in which HIF activity appears normal. This observation suggests that an alternative pVHL target is the causal feature predisposing VHL patients to development of pheochromocytoma [26]. In fact, further evidence has mechanistically linked pheochromocytoma in VHL disease to the other familial pheochromocytoma syndromes, as discussed in further detail below. pVHL is an ubiquitous protein with many distinct functions, perhaps accounting for its diverse tissue specificity and complex array of clinical manifestations when its gene becomes mutated.

13.4 Familial Paraganglioma/ Pheochromocytoma Syndromes

One of the more recently described genetic entities, familial paraganglioma/pheochromocytoma syndrome (PGL), is associated with development of pheochromocytoma and involves mutations in the genes which encode three of the four subunits of the succinate dehydrogenase (SDH) mitochondrial enzyme complex II [31]. SDH, a mitochondrial tumor suppressor, is a tricarboxylic acid (TCA) cycle enzyme that converts succinate to fumarate; it also acts as complex II of the electron transport chain [30]. As seen in VHL disease, mutations in the genes encoding this complex lead to stabilization of the HIF protein [30, 32, 33]. This HIF stabilization occurs through accumulation of succinate, which then inhibits the prolyl hydroxylation of HIF, a step necessary to target HIF for degradation by pVHL (Fig. 13.2) [30, 34]. This process leads to a state of pseudohypoxia in the cell, a condition shared by many cancers characterized by disruption in various oncogenes and tumor suppressor genes, including another component of the energy metabolism, fumarate hydratase (FH) [30, 35, 36].

SDH comprises four subunits, A, B, C, and D. Subunits A and B represent the catalytic portion of complex II, while subunits C and D anchor the complex to the mitochondrial membrane [37]. To date, mutations encoding for subunits B, C, and D of the SDH complex have been implicated in the development of paraganglial tumors [38–40]. In contrast, SDHA mutations have not yet been described as part of any known pheochromocytoma syndrome.

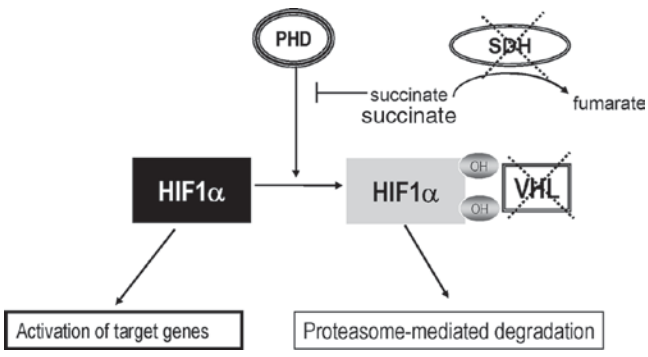

Fig. 13.2 HIF activation is a common feature of pheochromocytomas with *SDH* and *VHL* mutations. Loss-of-function mutations in *SDH* genes result in accumulation of succinate, which inhibits activity of prolylhydroxylases (PHD). PHDs are responsible for hydroxylation of specific HIF residues that are required for recognition by VHL for binding and proteasome-mediated degradation. Mutant VHL is unable to target HIF for degradation. Thus, *SDH* and *VHL* mutations result in increased availability of HIF and its downstream targets. VHL type 2C mutants are exceptional in which their ability to signal HIF for degradation is maintained. Other functions of VHL are likely to be responsible for tumor formation in these variants

Four distinct PGL syndromes have been recognized: types 1, 2, 3, and 4. The predisposition genes for types 1, 3 and 4 have been identified whereas the PGL type 2 susceptibility gene has *not* yet been defined [41, 42, 77]. While these mutations are inherited in an autosomal dominant pattern, resultant disease penetrance is variable [32]. PGL type 1 disease, characterized by mutations encoding SDHD, results most often in benign head and neck paragangliomas [43, 44], although malignancies have also been reported in patients with *SDHD* mutations [45–47]. Abdominal pheochromocytomas have also since been described in association with this entity [44]. The mode of inheritance seen in PGL type 1 disease has been thought to follow a pattern of maternal imprinting, as, until 2008, all reported cases of PGL type 1 were inherited from a paternal mutation carrier [43]. However, the molecular basis for the imprinting has been unclear, as SDHD shows biallelic expression, and the SDHD gene locus (11q23) is not a known locus of imprinted gene(s), leading to postulation that an alternative pathway for parent-of-origin inheritance exists [43]. The genetic pattern of maternal imprinting was further questioned when a single case demonstrating maternal transmission of an SDHD mutation was recently reported [48]. At this point, the confirmation or definitive exclusion of imprinting in association with SDHD requires further studies.

PGL type 3 disease, caused by mutations in the SDHC subunit, is almost exclusively associated with benign parasympathetic paraganglia of the head and neck (HNPs). However, these mutations are the least common of the PGL syndromes, and data revealing their intricacies are scarce. Based on the International Head and Neck Paraganglioma (HNP) Registry, *SDHC* mutations comprise approximately 4% of cases of HNP [42]. In conjunction with this registry is the Pheochromocytoma Registry, which found no cases of adrenal or extraadrenal pheochromocytoma related to *SDHC* mutations in 371 unrelated patients with adrenal or extra-adrenal pheochromocytoma [42, 49]. However, two recent case reports illustrate patients presenting with pheochromocytoma and *SDHC* mutations [49, 50]. The types of mutations seen in PGL type 3 disease are classic single base substitutions or more complex, involving large deletions that were previously undetected. Identification of the latter in part explains the apparently low incidence of PGL type 3 reported in previous studies that used direct sequencing as the mutation screening method.

PGL type 4, characterized genetically by mutations or deletions in SDH subunit B, typically presents clinically with extra-adrenal pheochromocytoma. Interestingly, mutations in *SDHB* account for the largest part of "apparently sporadic" tumors found to carry a germline mutation in any pheochromocytoma susceptibility gene [32]. However, HNP and adrenal pheochromocytoma may also be seen. Patients harboring *SDHB* mutations demonstrate high risk of malignant disease

[51]. In addition to greater risk of malignancy, *SDHB* mutation carriers have shortened survival when compared to other patients with malignant disease at presentation [52]. The reasons for the association between *SDHB* mutations with aggressive disease are unclear at present. Recent in vitro work based on short-interfering (si) RNA has suggested that cells with down-regulated SDHB display increased adhesion properties [53]. Future studies should address whether these features affect the intrinsic aggressiveness of mutant SDHB tumors or other yet unidentified targets of the mutation account for this unique phenotype.

13.5 Neurofibromatosis Type 1

Neurofibromatosis type 1 (NF1) (von Recklinghausen disease), an autosomal dominant inherited disease characterized by multiple neurofibromas, hamartomas of the iris, café-au-lait spots, and axillary or inguinal freckling, may also be accompanied by pheochromocytoma in some patients, generally 0.1–5.7% of cases. One of the most common inherited syndromes, NF1 carries 100% disease penetrance within families. However, the clinical spectra of the disease vary dramatically even within families [54]. Clinical disease develops when the *NF1* tumor suppressor gene, comprising 57 coding exons, is mutated. Genetic analysis of pheochromocytoma in the setting of NF1 is complex as a result of the gene's large size, the presence of pseudogenes, and the relative low frequency of the pheochromocytoma phenotype. An additional complicating factor is that mutations are randomly distributed across all coding portions of the gene such that a genetic "hot spot" does not exist in the *NF1* gene [55]. There has been some prior debate as to whether the development of pheochromocytoma is purely coincidental in these patients, as the frequency is low; however, the prevalence amongst NF1 patients remains greater than that found in the general population. In addition, Bausch et al. studied 37 patients with both NF1 and pheochromocytoma and reported loss of heterozygosity (LOH) at the *NF1* locus on chromosome 17q11.2 in 67% of the 21 blood-tumor pairs analyzed. This is in contrast to sporadic pheochromocytoma in which LOH at this region is not typically identified. These data suggest that loss of the *NF1* gene actively contributes to the pheochromocytoma pathogenesis in patients with neurofibromatosis type 1 [55].

The *NF1* gene encodes, neurofibromin, a protein with a GTPase Activating Protein (GAP) domain [56]. Its major function in acting as a tumor suppressor is in stimulation of conversion of the active GTP-bound RAS to the *inactive* GDP-bound RAS. By rendering RAS inactive, the typical proto-oncogenic signaling of RAS is terminated [54]. A microarray-based gene expression profiling study done by Powers et al. showed that *NF1* knockout mice with pheochromocytoma have overexpression of many genes responsible for early development of the central and peripheral nervous systems including the RET receptor tyrosine kinase [57]. When ligand binding of the receptor tyrosine kinase occurs, dimerization and autophosphorylation allow it to recruit adaptor proteins responsible for the activation of RAS [54]. When the *NF1* gene is mutated, RET is overexpressed and RAS stays activated leading to downstream signaling causing further cell growth and differentiation. An important component of this signaling is mTOR, which has been shown to be upregulated in *NF1*-deficient tumors via the activation of RAS in conjunction with PI3 kinase [58–60]. While the incidence of malignant pheochromocytoma in NF1 disease is low [55], NF1 patients are at risk for development of additional malignancies including peripheral nerve sheath tumors and gliomas. The initial discovery of RAS activation in NF1 prompted study of the effects of RAS inhibitors on tumor growth. However, little success has been achieved [61]. In contrast, the use of rapamycin, an mTOR inhibitor, has demonstrated therapeutic promise in early investigation of NF1-related malignancies [59, 62].

13.6 A Common Defect?

An intriguing outstanding question in the field of pheochromocytoma-paraganglioma remains the genetic diversity (at least six susceptibility tumors) contrasted with relative homogeneity in clinical phenotype. This pattern implies that the apparent genetic distinction may translate into overlapping signals or pathway cross talk. Using expression profiling in a large series of hereditary and sporadic pheochromocytomas and paragangliomas, two unique transcription groups ("clusters") were identified (Fig. 13.3) [33]. "Cluster 1" includes those tumors with *VHL*, *SDHB*, or *SDHD* mutations, suggesting that hypoxia, extracellular matrix components, and angiogenetic factors unify this group. In contrast, "cluster 2" encompasses *RET* and *NF1* mutations, indicating gene transcription associated with RNA synthesis and processing and also tyrosine kinase signaling [31, 33].

More recently, Lee et al. demonstrated that a common molecular mechanism underlying the development of all forms of familial pheochromocytoma may exist (Fig. 13.3). In embryological development of sympathetic neuronal precursors, cells undergo apoptosis when nerve growth factor (NGF) becomes limited. This apoptosis is c-Jun dependent but can be antagonized by JunB. In addition, a member of the prolyl hydroxylase family, EglN3 also helps regulate apoptosis. EglN3 is shown to act downstream of c-Jun, and it is sensitive to SDH activity [26]. When considering the molecular mechanisms underlying VHL and SDH-related

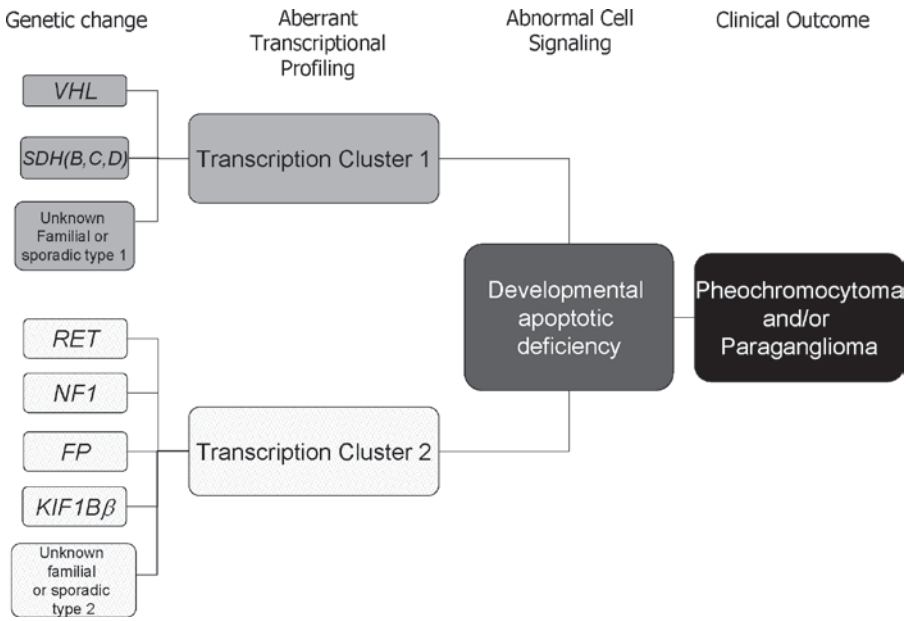

Fig. 13.3 Diagrammatic display of the genotypes and various levels of phenotypic changes (transcriptional, cellular and clinical) related to pheochromocytoma and paragangliomas. The mutations that lead to pheochromocytoma, including recently reported *KIF1Bβ* gene changes, converge into two transcriptional groups, characterized by pseudohypoxic activation (type 1) or increased tyrosine kinase signaling (type 2), amongst other transcriptional changes. Sporadic tumors and uncharacterized familial tumors are also components of the two major transcriptional clusters. Defective apoptosis of chromaffin precursor cells may be the end result of these mutations and lead to abnormal cell growth which can in turn progress to pheochromocytoma or paraganglioma

pheochromocytoma, HIF emerges as a possible link. However, although this has been clearly shown to be a prominent signal in both variants of pheochromocytoma, it does not explain all subtypes of mutant tumors. As described above, type 2C VHL disease has normal levels of HIF activity, indicating that an alternative pVHL target is responsible for the development of pheochromocytoma. Lee et al. found that all pVHL mutants fail to down-regulate JunB and, as a result, c-Jun decreases, and apoptosis is limited during NGF withdrawal [26]. *SDH*, when mutated, leads to accumulation of succinate, which then causes inhibition of the prolyl hydroxylases including EglN3, shown above to be required for neuronal apoptosis. Similar to *VHL* and *SDH* mutants, *NF1* and *RET* mutations seem to play a role in altering neuronal precursor apoptosis. When the NF1 tumor suppressor is lost, NGF-independent survival of neurons occurs, as NF1 normally inhibits downstream signaling via the NGF receptor TrkA. Similarly, RET is the glial cell line-derived growth factor (GDNF) receptor and can cross-talk with the NGF receptor TrkA [63]. In addition, when RET is mutated, JunB is upregulated, and apoptosis is limited. All of these findings implicate abnormal signaling in the neuronal apoptotic pathway during development as a converging defect in familial pheochromocytoma (Fig. 13.3). When families harbor germline mutations in *RET*, *VHL*, *SDH*, or *NF1*, apoptosis is limited during embryogenesis of neuronal precursors. These abnormally surviving cells may become the original focus for neoplastic transformation later in life [26].

13.7 Other Familial Forms of Pheochromocytoma

While there are six well-known pheochromocytoma susceptibility genes, as described above, there are also familial clusters of the disease and bilateral cases of pheochromocytoma for which the susceptibility gene has not been identified [64, 65]. Recently, two novel susceptibility loci, the familial pheochromocytoma (FP) loci, were described using integrative genomics [66]. A large Brazilian-Portugese family with six members afflicted with pheochromocytoma was studied. Using linkage and haplotype analysis, two loci at chromosomes 2 and 16 emerged. As LOH was seen at the chromosome 2 locus, the classic tumor-suppressor gene model was hypothesized. At the locus on chromosome 16 however, there was no LOH, and the area identified actually overlapped with the area linked to familial neuroblastoma, another sympathoadrenal tumor [67]. The novel FP loci, when included in the expression profiling described above, tracked with the *RET* and *NF1* mutants, indicating probable similarities in their signaling properties [66].

Another gene recently associated with the development of pheochromocytoma is the tumor suppressor *KIF1Bβ* on chromosome 1p36.2, a region commonly deleted in neural crest-derived tumors [68]. As discussed previously, the proposed common molecular pathway in pheochromocytoma involves limited apoptosis in response to NGF withdrawal during embryogenesis, and the prolyl hydroxylase, EglN3 is a key enzyme in this setting [26]. KIF1Bβ has been shown to act downstream of EglN3 as another proapoptotic factor, and loss-of-function germline mutations of *KIF1Bβ* have been identified in some cases of pheochromocytoma and also neuroblastoma [68]. This gene was later studied in the context of a cancer-prone family with five individuals affected by tumors of both neural and non-neural origin including pheochromocytoma, neuroblastoma, ganglioneuroma, and lung adenocarcinoma [69]. Microarray-based expression profiling was again utilized, and these *KIF1Bβ* mutants clustered with *NF1* and *RET* pheochromocytomas, implying transcripts involving signaling in the RAS/MAPK pathway. While the *KIF1Bβ* gene is characterized as a tumor-suppressor, LOH was not seen in this particular family, a deviation from the classic two-hit model. The possible spectrum of disease seen with *KIF1Bβ* mutation remains unknown, and more data are needed prior to establishing a definitive causal relationship between the *KIF1Bβ* gene and pheochromocytoma.

13.8 Sporadic Pheochromocytoma/ Paraganglioma

While 25–30% of pheochromocytoma cases are familial, the majority remain apparently sporadic [31]. However, several published large series of pheochromocytoma patients reveal that germline mutations in the known susceptibility genes of *RET*, *VHL*, *SDHB*, and *SDHD* may be seen in up to 24% of "sporadic-appearing" cases [44, 70]. Additional studies have not been able to replicate these numbers, but it is now well-established that 8–16% of sporadic-appearing pheochromocytomas are due to an unsuspected germline mutation in one of the susceptibility genes [32, 71]. Less than 5% of pheochromocytomas are believed to carry somatic mutations in any of the pheochromocytoma susceptibility genes. These numbers reinforce the "developmental hypothesis" to explain the origin of pheochromocytoma, described above, and underscore the likelihood that additional susceptibility genes do exist.

13.9 Challenges and Future Directions

As demonstrated, a growing number of pheochromocytomas and paragangliomas are linked to inherited mutations. The commonly cited incidence of 25–30% is likely an underestimation given the addition of several novel susceptibility genes including the *FP loci*, *KIF1Bβ*, and perhaps others *not yet* discovered. In addition, it has been reported that some cases of pheochromocytoma/paraganglioma are characterized genetically by large deletions or chromosomal rearrangements rather than typical point mutations [72]. These types of defects are not readily detected using exon-based direct sequencing but instead require alternative technical approaches. Cascon et al. described three patients with gross deletion of the *SDHB* gene, discovered by multiplex ligation-dependent probe amplification (MLPA) or quantitative real time PCR [73, 74]. As utilization of these techniques increases, additional familial forms of pheochromocytoma/paraganglioma will likely be identified, increasing the incidence of heritable disease.

Greater insight into the biology of pheochromocytoma has been achieved in recent years. As we now know that at least one-quarter of cases represent inherited forms of the disease, and a significant number of apparently sporadic cases actually harbor mutations in pheochromocytoma susceptibility genes, genetic screening should probably be utilized more frequently than previously thought [75, 76]. While it is not cost-effective to screen every patient with pheochromocytoma or paraganglioma, a careful family history should be taken to help determine which patients will benefit from testing. History regarding not only incidence of pheochromocytoma and paraganglioma, but also sudden cardiac death should be obtained, as many pheochromocytomas are only discovered on autopsy. The First International Symposium on Pheochromocytoma, held in 2005, recommends genetic testing for *VHL*, *RET*, *SDHB*, and *SDHD* in patients less than 50 years of age with a family history. In cases of multiple tumors at distinct anatomic sites, *SDHB*, *SDHD*, and *VHL* should be examined. Malignant pheochromocytoma warrants testing for *SDHB* and *VHL*, while bilateral tumors require testing for *RET*, *VHL*, and *SDHD* [20]. Refinement and updates on these guidelines will be driven by future studies based on detailed analyses of larger cohorts and progress on the genetics of these tumors.

Enormous strides have been made in understanding the molecular pathogenesis underlying pheochromocytoma and paraganglioma. These advances not only help guide clinical management of affected patients, but also offer valuable insight into possible therapeutic targets, particularly when surgical resection is *not* effective or possible. In addition, the investigation of the pheochromocytoma-susceptibility genes has revealed molecular intricacies, which may be applied to other more common forms of neoplasia.

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Chapter 14
Endocrine Ovaries

Xavier Matias-Guiu

14.1 Histology of the Ovary

14.1.1 Surface Epithelium and Epithelial Inclusion Cysts

The ovaries are covered by a single layer of modified peritoneal cells, which 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 as 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].

14.1.2 Stroma

The ovarian cortical stroma is composed of spindle-shaped cells arranged in whorls and storiform patterns. In women in the reproductive age group, the stroma contains follicles, corpus luteum, and corpus albicans. 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.

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14.1.3 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 400.000 at birth.

Primordial follicles are composed of a primary oocyte surrounded by a single layer of inconspicuous granulosa cells (Fig. 14.1). After gonadotropin stimulation, the oocyte enlarges and the granulosa cells become polygonal (primary follicle).

Follicular maturation produces changes in the morphology of follicles (secondary follicles). Granulosa cells proliferate and become stratified (pre-antral follicle), and the surrounding cortical stroma differentiate into both theca interna and externa. At the pre-antral stage, the enlarging oocyte is surrounded by an eosinophilic, 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 a few layers of granulosa cells and protrudes into the cavity, in an eccentric position (cumulus oophorus) (Fig. 14.2).

Although several follicles start the process of follicular maturation during each menstrual cycle, only the *dominant follicle* develops into a 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.

After ovulation, the disrupted mature follicle becomes 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 which originates capillaries that penetrate the granulosa cell layer and reach the central cavity. The central cavity of the corpus luteum is lined by an inner fibrous layer composed of fibroblasts that accompany the capillaries and a dense reticulum network. After 8–9 days, involution changes start; luteinized

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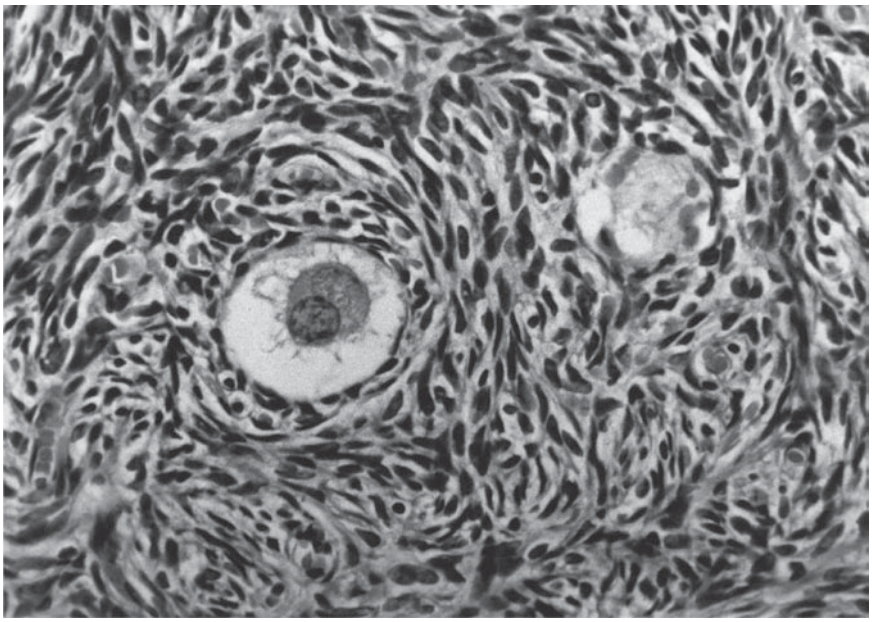

Fig. 14.1 Microscopical appearance of a primordial follicle. The oocyte is surrounded by a thin layer of flat granulosa cells

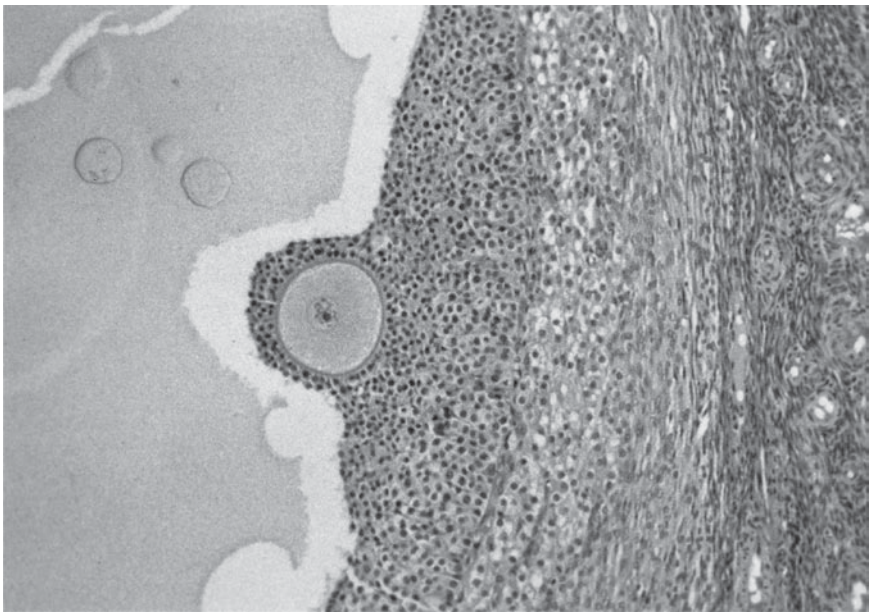

Fig. 14.2 A mature (graafian) follicle. The oocyte is surrounded by a few layers of granulosa cells, protruding into the follicular cavity

granulosa cells decrease in size, accumulate lipids, and show degenerative nuclear changes. There is progressive fibrosis and transformation into a hyalinized corpus albicans.

14.1.4 Other Structures

It is important to consider 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 post-menopausia.

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 ACTH stimulation. Rete ovarii is similar to the rete testis and consists of tubules with intraluminal projections.

14.1.5 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, CA12.5, and calretinin.

The vast majority of the other ovarian cell elements share some immunohistochemical markers, since all of them have the ability to produce steroid hormones. In general, any steroid hormone producing cell may exhibit positivity for alpha-inhibin, müllerian inhibiting substance, Melan-A (MART-1), and calretinin. Some cell types are also positive for CD99. These markers are very important in distinguishing between sex cord-stromal and steroid cell tumors from epithelial-stromal or metastatic neoplasms. By far, alpha-inhibin is the most useful of them [3] (Fig. 14.3).

Inhibin and inhibin-related peptides (activin and follistatin) are gonadal hormones involved in the 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 alpha subunit and a beta-A or beta-B subunit linked by disulfide bonds. In contrast, activins are homodimers of two beta subunits. The alpha 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 alpha-inhibin does occur but it appears to be limited to a few tissues, such as the adrenal gland, placenta, pituitary, and central nervous system.

In the ovary, alpha-inhibin immunoreactivity has been found to be very useful, particularly in distinguishing between sex cord-stromal tumors and carcinomas [5–8]. The possible use of alpha-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].

Alpha-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 non-neoplastic stromal cells that frequently surround clusters of neoplastic cells in the so-called ovarian tumors with functioning stroma, are frequently immunoreactive for alpha-inhibin [10].

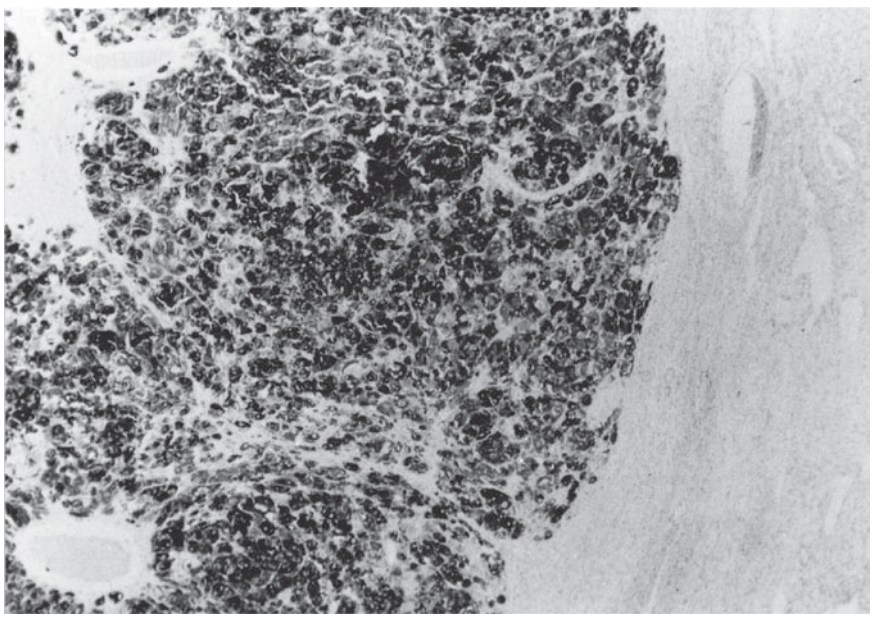

Fig. 14.3 Strong staining for alpha-inhibin in a Hilus cell tumor

14.2 Tumor-Like Lesions and Functional Cysts

14.2.1 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. 14.4). 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, which is now recognized to be secondary to mutations located in exons of GNAS1 that encode the alpha subunit of the stimulatory G protein ($G_s\alpha$) [12].

14.2.2 Corpus Luteum Cyst

Corpus luteum cyst is a cystic corpus luteum with a diameter of more than 3 cm. It may be associated with menstrual irregularities and, like follicle cysts, may rupture and cause intraabdominal hemorrhage (Fig. 14.5).

14.2.3 Ovarian Remnant Syndrome

The ovarian remnant syndrome is a clinico-pathologic 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.

14.2.4 Polycystic Ovarian Syndrome (POS)

POS is a clinicopathologic disorder of unknown, probably heterogeneous etiology, characterized by chronic anovulation, hyperandrogenism, and enlarged polycystic ovaries. POS affects between 5 and 10% of women of reproductive age [13,14,15,16].

An international consensus group proposed that POS can be diagnosed after the exclusion of other medical conditions that cause irregular menstrual cycles and androgen excess, and that the determination that at least two of the following features are present: (1) oligoovulation or anovulation; (2) hyperandrogenemia, (3) clinical manifestations of hyperandrogenism, and (4) polycystic ovaries as defined by ultrasonographic examination [17]. According to this consensus effort, the presence of enlarged ovaries with many subcapsular cysts, by echography or gross inspection is not a necessary requirement for the diagnosis of POS. In some cases, the 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.

From a pathologic viewpoint, POS is characterized by a bilateral enlargement of ovaries with multiple subcapsular

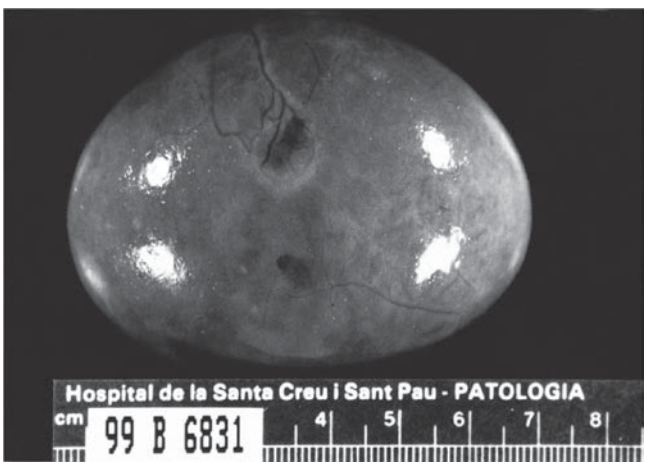

Fig. 14.4 Gross appearance of a follicle cyst in a neonate

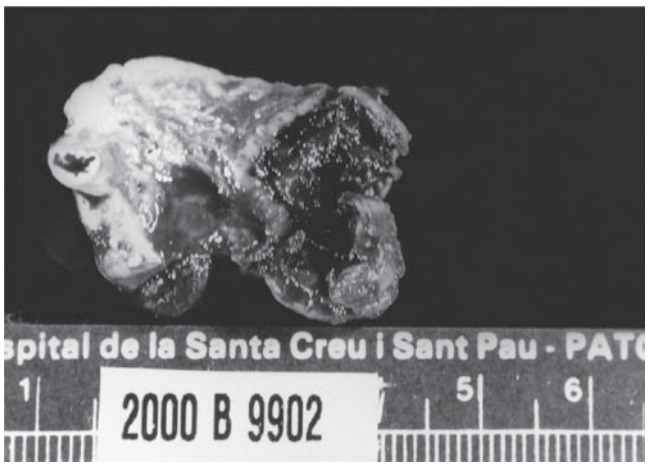

Fig. 14.5 Gross appearance of a corpus luteum cyst that ruptured and caused an intraabdominal hemorrhage

follicle cysts (Figs. 14.6 and 14.7), 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. 14.8).

Although hyperandrogenism is the most characteristic endocrine manifestation of POS, several patients present with hyperestrogenism as a result of the transformation of androgens into estrogens by aromatization in peripheral fat [18,19]. In these cases, hyperestrogenism may result in the development of hyperplasia or carcinoma of the endometrium, particularly in pre-menopausal patients.

Determining the etiology of POS has proven elusive, and no single etiologic factor fully accounts for the spectrum of alterations in POS. In vitro and in vivo studies on ovarian theca cells from patients with POS show greater efficiency in converting androgenic precursor to testosterone than normal theca cells. Moreover, women with POS have increased luteinizing hormone pulse frequency. Finally, it is clear that insulin resistance plays a role in the pathogenesis of this disorder [20, 21].

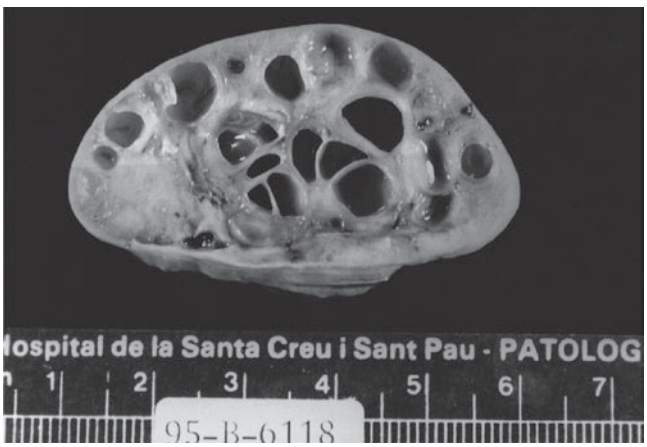

Fig. 14.6 Gross appearance of a gonad in a patient with polycystic ovarian syndrome

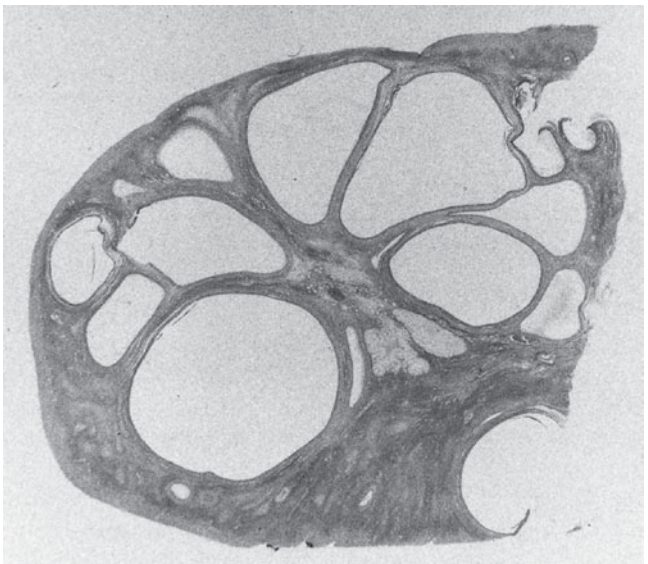

Fig. 14.7 Low power view of a polycystic ovary

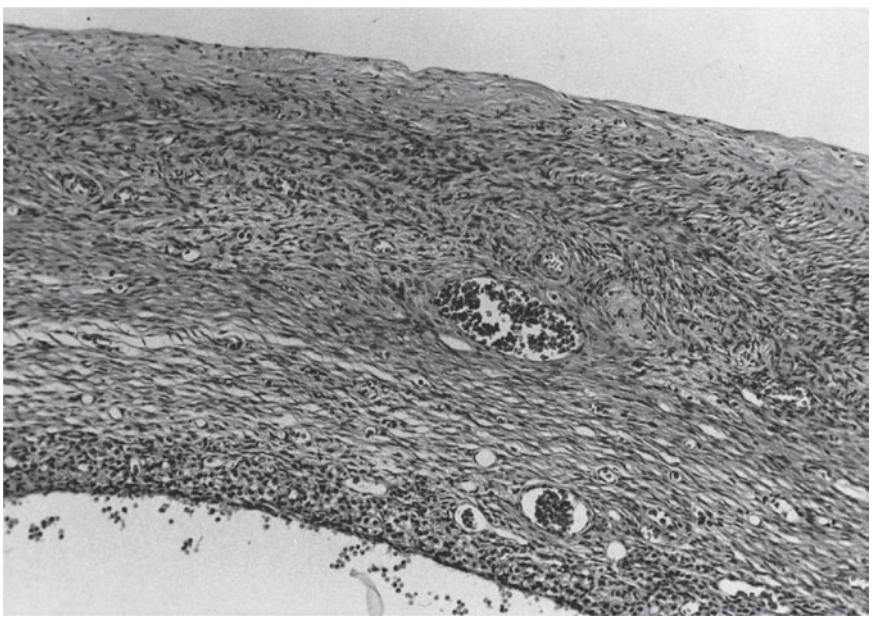

Fig. 14.8 Microscopic appearance of a polycystic ovary. A follicle cyst is lined by a thin granulosa cell layer and a prominent theca cell layer. Note the presence of hyalinization in the subcapsular connective tissue

14.2.5 *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. 14.9).

Both stromal hyperplasia and stromal hyperthecosis may occur in pre- and post-menopausal women. In young women, stromal hyperthecosis exhibit many similarities with POS. In fact, some patients may show overlapping features between both entities. In these patients, stromal hyperthecosis usually manifests with hyperandrogenism, but hyperestrogenism may occur with development of endometrial hyperplasia or carcinoma (Fig. 14.10). Like POS, stromal hyperthecosis is somehow related to insulin resistance, and hence, is usually associated with acanthosis nigricans in the skin (the so-called HAIR-AN syndrome) [22].

In post-menopausal women, stromal hyperplasia and hyperthecosis may be associated with steroid hormone production. In some cases, the hirsutism and virilization may be even more severe than that associated with sex cord-stromal or steroid cell tumors. On the other hand, it has

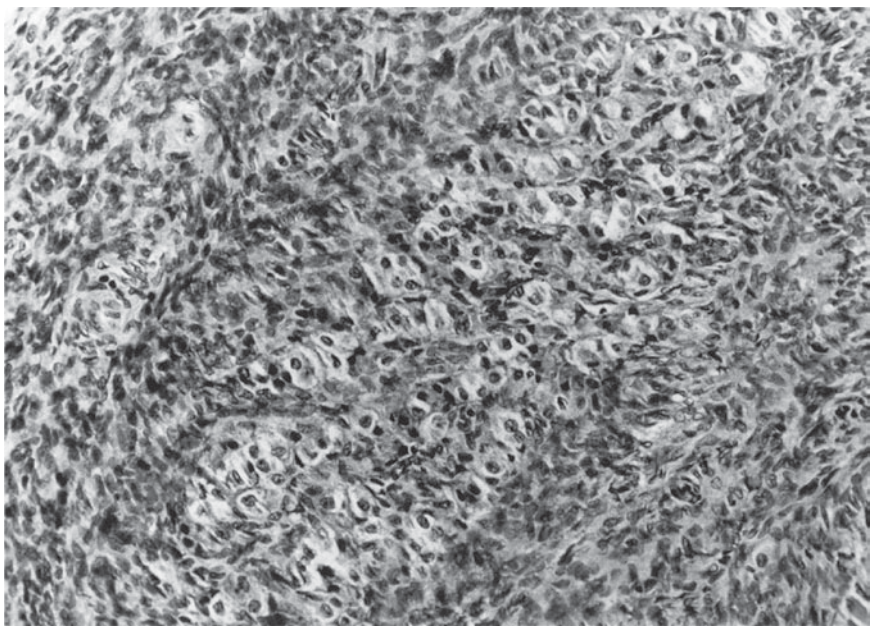

Fig. 14.9 Numerous luteinized stromal cells in ovarian hyperthecosis

Fig. 14.10 An endometrioid carcinoma of the endometrium in a pre-menopausal woman with bilateral ovarian hyperthecosis

been suggested that stromal hyperplasia and hyperthecosis may be the initial source for steroid hormone production in post-menopausal women with hyperestrogenism. It has been shown that the ovarian stroma of patients with endometrial carcinoma frequently contain clusters of luteinized stromal cells.

14.2.6 *Massive Edema and Fibromatosis*

Massive edema is characterized by the enlargement of one or two ovaries, because of the accumulation of edema fluid. Massive edema is usually secondary to torsion (Figs. 14.11 and 14.12). In some cases, stromal lutein cells may become

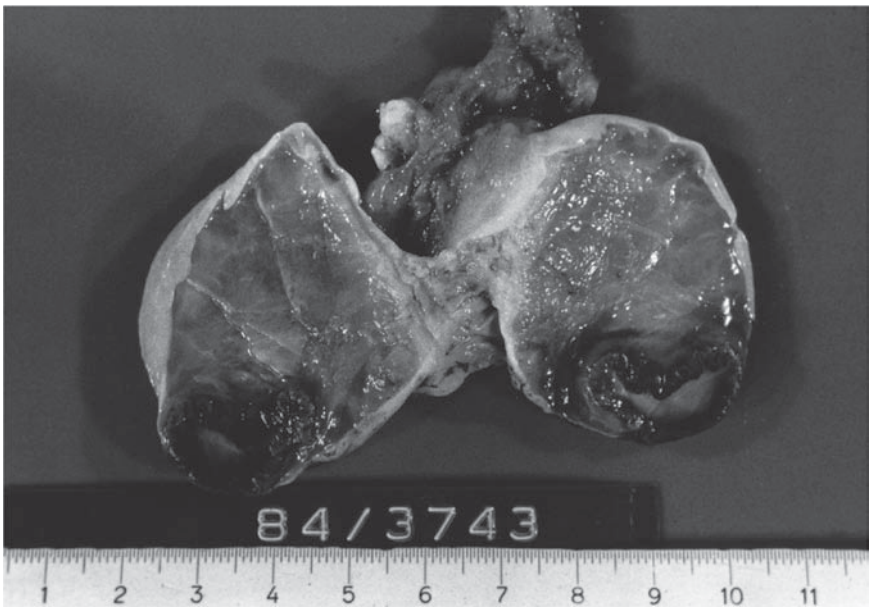

Fig. 14.11 Gross appearance of Massive Edema. The ovary is enlarged and contains abundant watery fluid

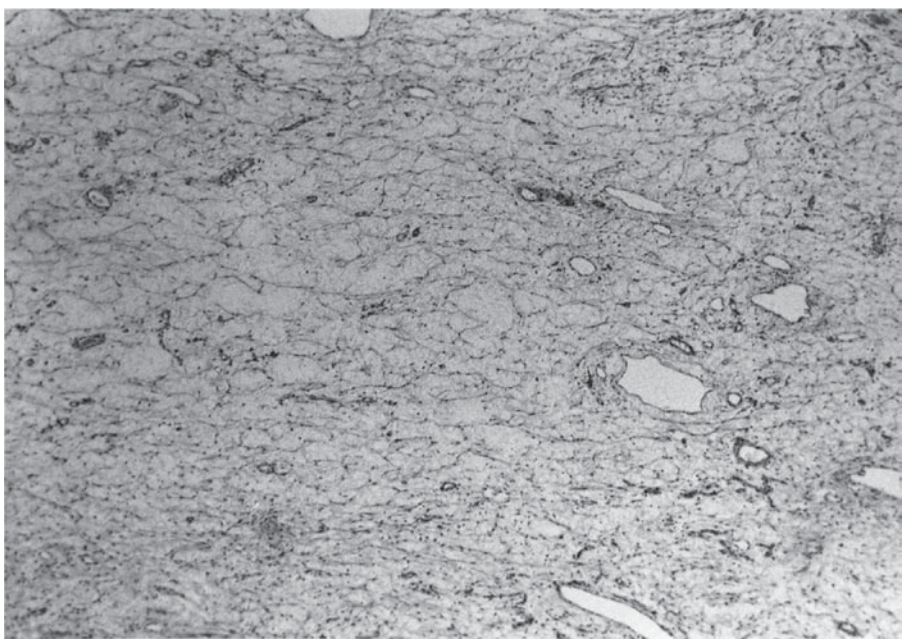

Fig. 14.12 Microscopic appearance of ovarian massive edema

activated, and the patients may present 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].

14.3 Ovarian Lesions Associated With Pregnancy

14.3.1 Corpus luteum of Pregnancy

The corpus luteum of pregnancy is very similar to that of menstruation. However, it shows two distinct histological features: eosinophilic hyaline bodies (Fig. 14.13) and calcification [24].

14.3.2 Pregnancy Luteoma

Pregnancy luteoma is a tumor-like 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 tumor-like lesion that regresses spontaneously after termination of pregnancy [24].

14.3.3 Hyperreactio Luteinalis

Hyperreactio luteinalis is also a tumor-like lesion that occurs in pregnant patients with high hCG levels (for example, 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].

14.3.4 Large Solitary Luteinized Follicular Cyst of Pregnancy and Puerperium

It is a unilateral and unilocular cyst that contains watery fluid, frequently detected at the time of cesarean section or during routine physical examination of puerperium. It is characteristically lined by large, eosinophilic, luteinized cells with bizarre nuclei. It typically occurs during pregnancy or puerperium (Figs. 14.14 and 14.15). It can reach a large

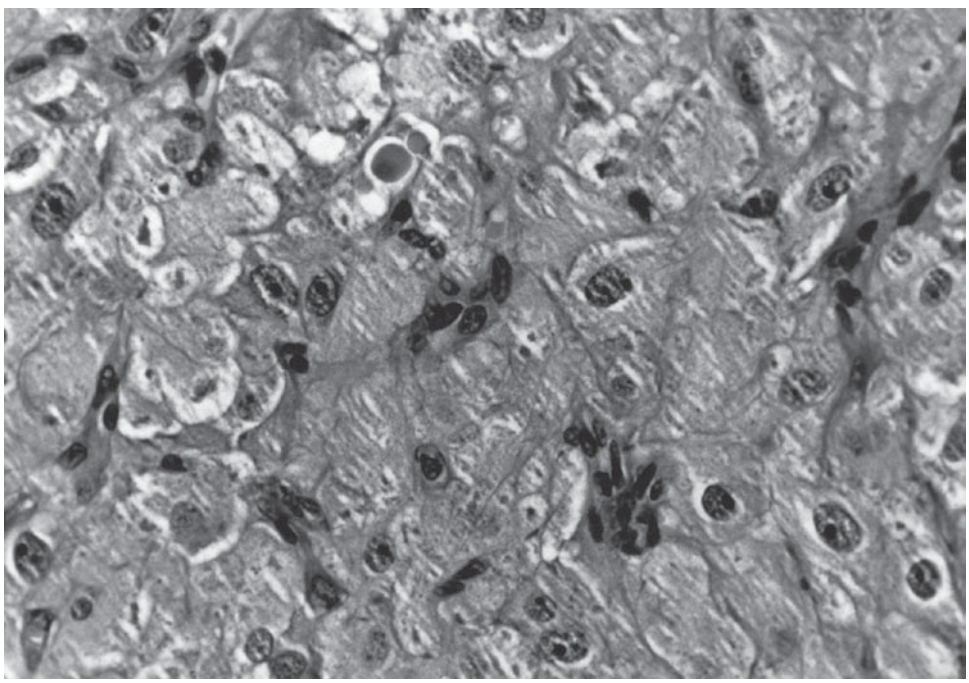

Fig. 14.13 Eosinophilic hyaline bodies in a corpus luteum of the pregnancy

size and pose problems with malignancy. The major important differential diagnosis is with a unilocular cystic granulosa cell tumor [25].

14.3.5 Granulosa Cell Proliferation of Pregnancy

They are characterized by clusters of granulosa cells, frequently associated with atretic follicles. They are incidental findings, not associated with endocrine manifestations, but they may pose problems in differential diagnosis [24, 26].

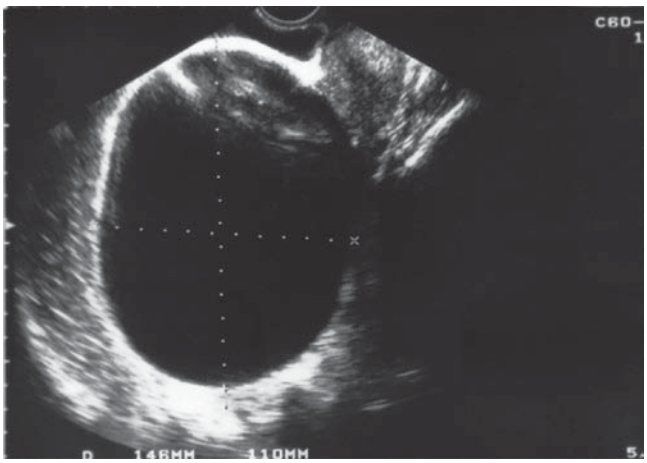

Fig. 14.14 Ultrasonographic scan of a large solitary luteinized follicular cyst of pregnancy and puerperium

14.4 Endocrine Syndromes Associated with Ovarian Tumors

The ovarian neoplasms may be accompanied by a great variety of endocrine manifestations. By far, hyperandrogenism and hyperestrogenism are the most frequent of them [27].

The normal ovary contains a great 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 some 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 the ovarian tumors reflects overlapping in the production of steroid hormones in the normal cell counterparts. Some tumors are associated with hyperestrogenism, while others show hyperandrogenism, but the vast majority may be associated with both types of hormone excess.

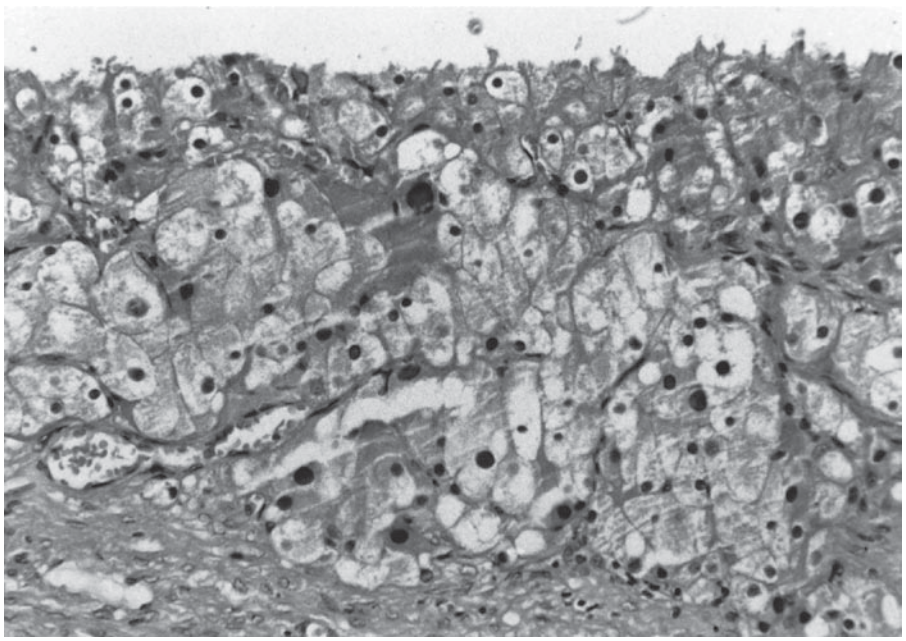

Fig. 14.15 Microscopic appearance of a large solitary luteinized follicular cyst of pregnancy and puerperium; prominent luteinization and bizarre nuclei

14.4.1 Hyperestrogenism

The ovarian tumors most frequently associated with hyperestrogenism are thecoma, granulosa cell 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 pre-menopausal women can present menstrual irregularities or amenorrhea. Post-menopausal women typically have vaginal bleeding; in these patients, an endometrial biopsy may demonstrate the presence of endometrial hyperplasia, or cervico-vaginal smears can show an abnormal estrogenic pattern.

14.4.2 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-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, androstenedione may be elevated. In contrast to virilizing adrenal tumors, the urinary 17-ketosterone are usually (but not always) normal, and the serum levels of dehidroepiandrostenedione sulfate are usually normal.

It is worth emphasizing that, particularly in post-menopausal women, hyperandrogenism with hirsutism and virilization may be due to non-neoplastic disorders such as stromal hyperthecosis or massive edema.

14.4.3 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 are consistent with steroid hormone secretion, and are 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 (for example, 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 metastasis 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. 14.16) or contain clusters of luteinized stromal cells (Fig. 14.17). 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 clinical evidence of hormone excess. For example, sometimes, patients with Krukenberg tumors present with virilization as initial symptom, as a result of the activation of the ovarian stroma, leading to a pre-operative mis-interpretation as a sex cord-stromal ovarian tumor [27].

The reason for the activation of stroma in the third group of OTFS is unclear. Several investigators suggested that the ectopic production of hCG by tumor cells could explainthe 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.

14.4.4 Hypercalcemia

Hypercalcemia is a frequent paraneoplastic syndrome in tumors from different organs. Hypercalcemia 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 pre-existing

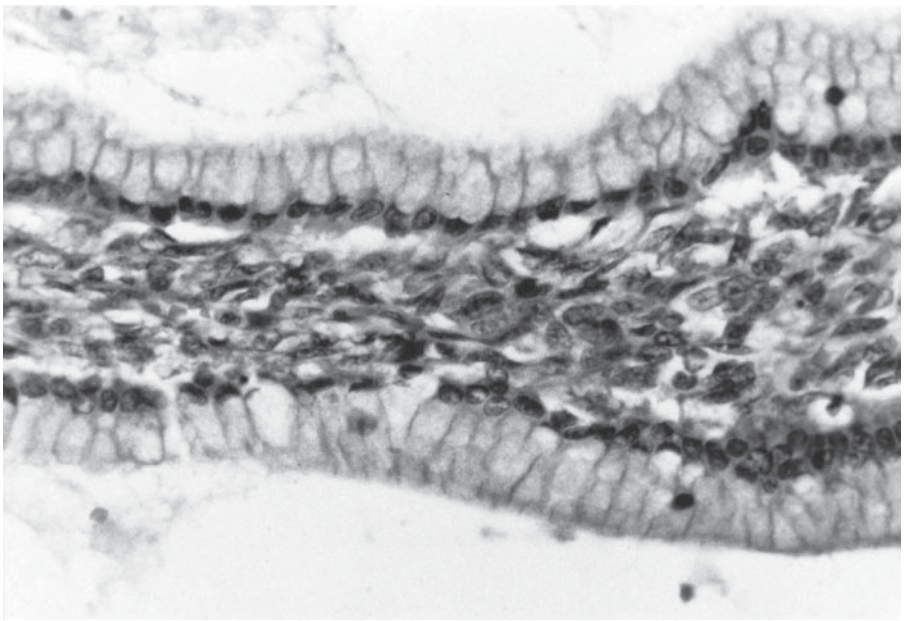

Fig. 14.16 Stromal luteinization in a mucinous cystadenoma with functioning stroma

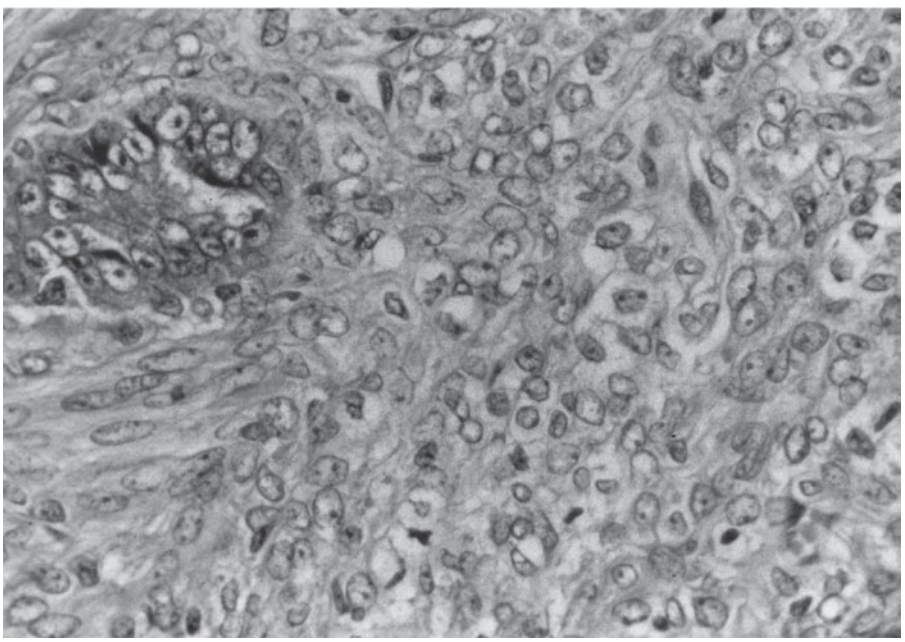

Fig. 14.17 Luteinized stromal cells in an endometrioid carcinoma with functioning stroma

dermoid cysts have also been reported in association with hypercalcemia.

The small cell carcinoma of the ovary is a distinct 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 patients. It is characterized by poorly differentiated small cells with densely hyperchromatic nuclei and scanty cytoplasm (Fig. 14.18); larger cells with abundant cytoplasm may be observed in approximately half of the cases. Distinctive follicle-like structures containing eosinophilic fluid may be present, which may pose problems in differential diagnosis with juvenile granulosa cell tumors. The lack of typical follicles of granulosa cell tumor, absence of a thecal component, higher mitotic rate, and negativity for alpha-inhibin are useful tools in differential diagnosis.

Attempts to demonstrate PTH immunostaining in these tumors were unsuccessful in the vast majority of cases [31].

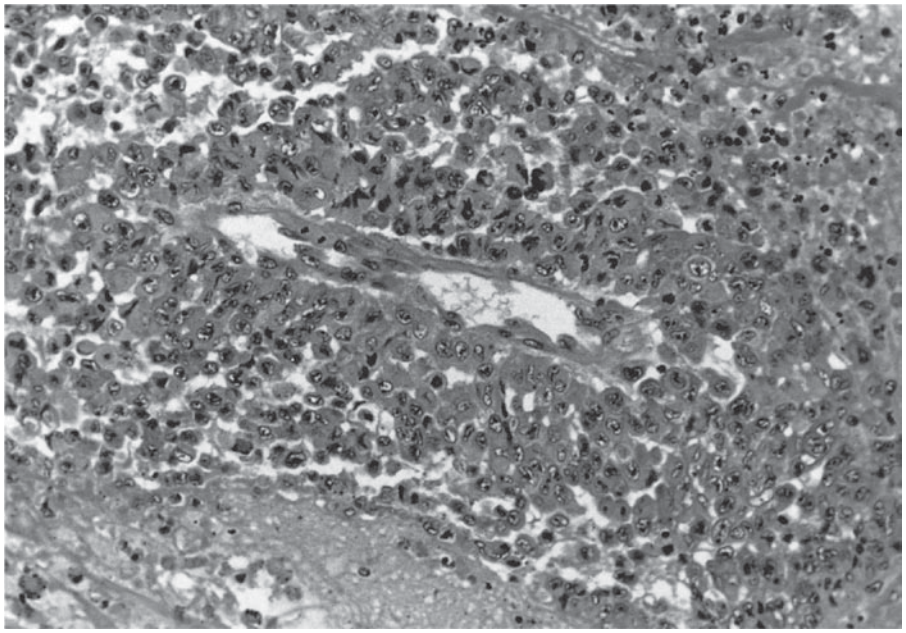

Fig. 14.18 Microscopic appearance of a small cell carcinoma associated with hypercalcemia

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 responsible for the hypercalcemia that is frequently associated with ovarian small cell carcinomas [32].

14.4.5 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]. As mentioned later on, steroid cell tumors may produce renin.

14.5 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.

14.5.1 Adult Granulosa Cell Tumor

Adult granulosa cell tumors (AGCT) 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 may present as a palpable mass, or as hemoperitoneum secondary to rupture. AGCT are usually unilateral, solid, and cystic. The solid component frequently shows a yellow discoloration [33, 34]. Occasional tumors are cystic and are frequently associated with virilization [35].

AGCT is composed of granulosa cells, but frequently contain theca or spindle stromal cells. Tumor cells are typically irregular and contain grooved nuclei (Fig. 14.19), haphazardly oriented to one another. Scattered cells with pleomorphic, large, atypical nuclei can be detected in approximately 2% of the tumors. AGCT may show a wide variety of architectural patterns which may be found separately or in combination. Mitotic rate is usually low. They include the microfollicular (the most characteristic), macrofollicular, insular, trabecular, solid-tubular, gyriform, and diffuse (sarcomatoid) components. Some tumors show rosette-like structures that resemble the Call-Exner bodies of the graafian follicles (Fig. 14.20). The presence of solid tubules resembling those of a well-differentiated Sertoli–Leydig cell tumor does not alter the diagnosis as long as they represent less than 10% of the tumor. Approximately, 2% of AGTC are mostly luteinized,and it is particularly strong in tumors occurring during pregnancy. Moreover, rarely, AGCT contain heterologous elements (rhabdomyoblasts or hepatic cells)

AGCT are potentially malignant tumors that may spread within the pelvis and lower abdomen. Recurrences usually appear within 5 years after the initial surgery. However, late recurrences and distant metastasis are sometimes

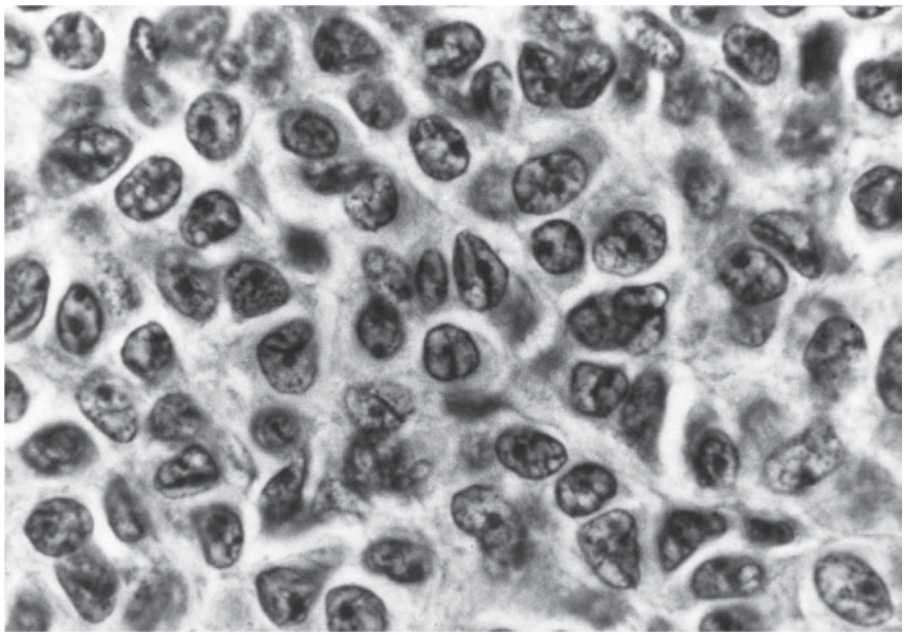

Fig. 14.19 Nuclear grooves in a typical adult granulosa cell tumor

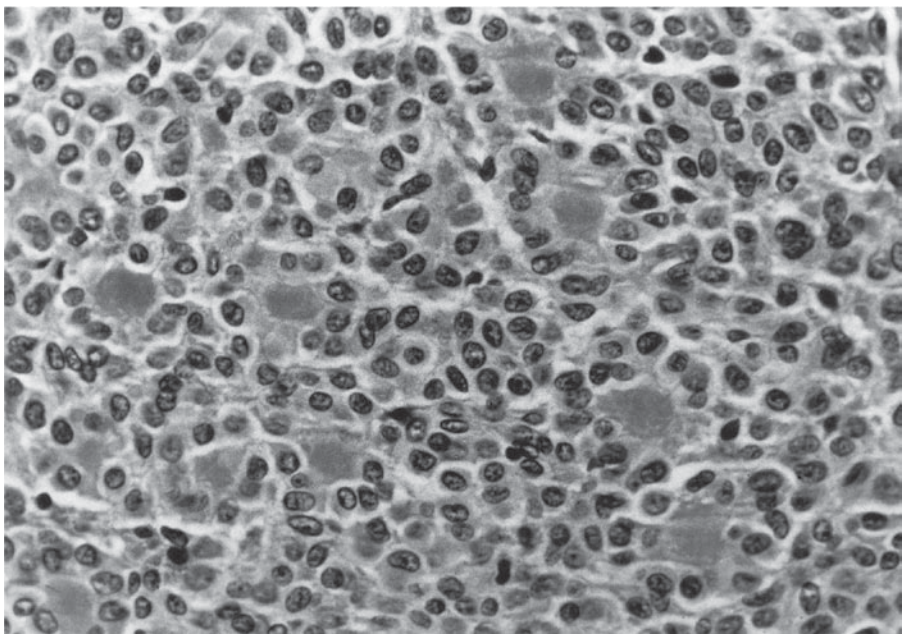

Fig. 14.20 Call-Exner bodies in an adult granulosa cell tumor

detected. The 10-year survival rate ranges from 60 to 90%. The prognostic factors have been recently reviewed [36]; the most important prognostic factor is stage, and the presence of metastasis or invasion of structures outside the ovary at the time of diagnosis. Less important adverse prognostic factors are tumor size, and possibly tumor rupture. An adverse prognosis has been attributed to the presence of a predominant diffuse or sarcomatoid component [36–38].

The most important differential diagnosis is with poorly-differentiated endometrioid carcinoma (Fig. 14.21); the presence of typical low-grade endometrioid carcinoma or adenofibromatous components, lack of nuclear grooves, and negativity for alpha-inhibin, are good tools for the correct diagnosis. Calretinin is highly sensitive but less specific. AGCT is negative for keratin 7 and EMA. Reticulin stains are also useful [8].

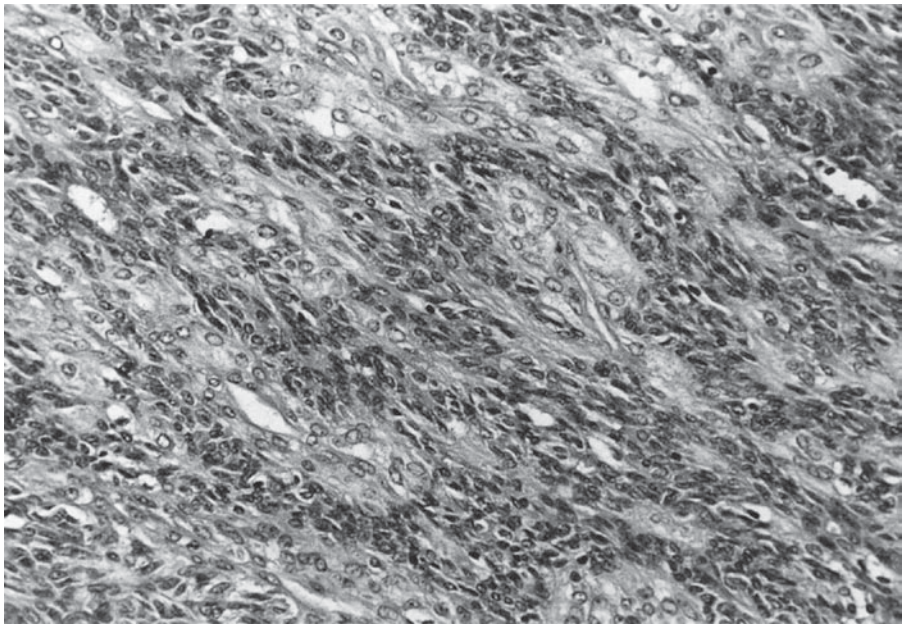

Fig. 14.21 Microscopic appearance of an ovarian endometrioid carcinoma that resembled a sex cord-stromal tumor. Tumor cells were negative for alpha-inhibin. Typical foci of low-grade endometrioid carcinoma were found after an intense sampling

14.5.2 Juvenile Granulosa Cell Tumor

Juvenile granulosa cell tumors (JGCT) are very rare and represent 5% of all granulosa cell tumors [39]. JGCT usually occurs in women less than 30 years of age, but it may occur in older patients also. When JGCT occurs before puberty, it may be accompanied by isosexual pseudoprecocity, because of estrogen secretion by tumor cells.

JGCT are usually unilateral, cystic, and solid. The microscopic picture is different from AGCT. Tumor cells are less mature, lack 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 that also occurs in children and young patients, and may show pseudo-follicular spaces. However, the appearance of cells and positivity for alpha-inhibin are good tools for diagnosis.

14.5.3 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. 14.22). Both cell elements may coexist in variable proportion in some tumors [11].

Typical thecomas usually occur in post-menopausal women, and are associated with estrogenic manifestations. Luteinized thecomas (thecomas with prominent luteinization) occur in younger patients and may be accompanied by androgenic symptoms. The luteinized thecomas associated with sclerosing peritonitis are bilateral, and the luteinized cells are typically smaller. Thecomas may be calcified. Malignant thecomas are very rare.

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 cord-like elements.

14.5.4 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 (SLCT). SLCT are unilateral (Fig. 14.23) and usually occur in women of reproductive age. They are usually associated

Fig. 14.22 Gross appearance of an ovarian fibroma

Fig. 14.23 Gross appearance of a Sertoli–Leydig cell tumor. In this case, the patient presented with hyperestrogenism and endometrial hyperplasia

with virilization, but may be non-functioning, or may even present estrogenic symptoms [42–44].

SLCT 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 non-specific fibrous stroma with nests of Leydig cells with occasional Reinke crystalloids [45].
2. Tumors of intermediate differentiation, which contain abortive tubules and cords of immature Sertoli cells, separated by a fibrous stroma and Leydig cells (Fig. 14.24).
3. Poorly differentiated or sarcomatoid tumors, composed of spindle cells with vague trabecular configuration.
4. SLCT with heterologous elements. These tumors contain foci of neoplastic cartilage, mucinous glands, neuroendocrine (carcinoid) cells, and skeletal muscle [46, 47].

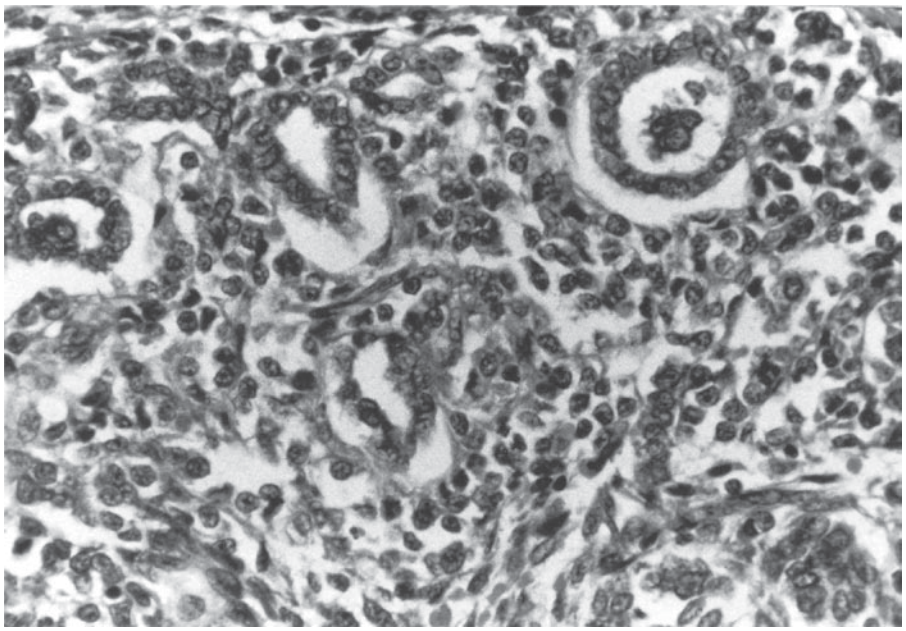

Fig. 14.24 Microscopic appearance of a Sertoli–Leydig cell tumor of intermediate differentiation

5. Tumors with retiform pattern. It has recently been established that retiform areas must comprise at least 90% of the tumor. Tumors with at least 10% but less than 90% are classified as SLCT with retiform elements. [48–50].

SLCT are usually stage Ia tumors that follow a benign behavior. Recurrence is associated with poorly differentiated tumors and the presence of mesenchymal heterologous elements.

14.5.5 Sex Cord Tumor with Annular Tubules (SCTAT)

SCTAT has a peculiar histologic appearance that is intermediate between granulosa and Sertoli cell tumor. It is composed of complex annular tubules that contain a central hyaline body of basement membrane material (Fig. 14.25). The vast majority of SCTAT are benign but a few are malignant [51].

SCTAT occurs in two settings: (1) as solitary neoplasms without evidence of any associated syndrome, which may follow a malignant course in at least 20% of cases; or (2) as a multifocal, bilateral tumor in association 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]. Peutz–Jegher-associated SCTAT are benign tumors

14.5.6 Gynandroblastoma

It is defined as a rare tumor that shows areas of granulosa-theca cell tumor, coexisting with others of Sertoli–Leydig cell tumor [50].

14.5.7 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 artefactual, due to DNA contamination [54, 55]. However, the potential involvement of pituitary gonadotropins, LH, and FSH in the development of sex cord tumors is still under investigation. Follitropin receptor knockout mice are associated with a high incidence of sex cord tumors [56]. Moreover, mice deficient in inhibin alpha-subunit gene develop granulosa and/or Sertoli cell tumors. [57]. The alpha-subunit of gip2, a G protein, has been found to be mutated in 30% of granulosa cell tumors [58], involving codon 179, and resulting in the substitution of either cysteine or histidine for arginine. DNA from Sertoli–Leydig cell tumors were found to lack the sex determining region Y gene (SRY) [59, 60]

Moreover, the fact that germline mutations in the STK11/LKB1 gene, on 10p13.3, were responsible for Peutz–Jegher syndrome [52] prompted some authors to investigate the

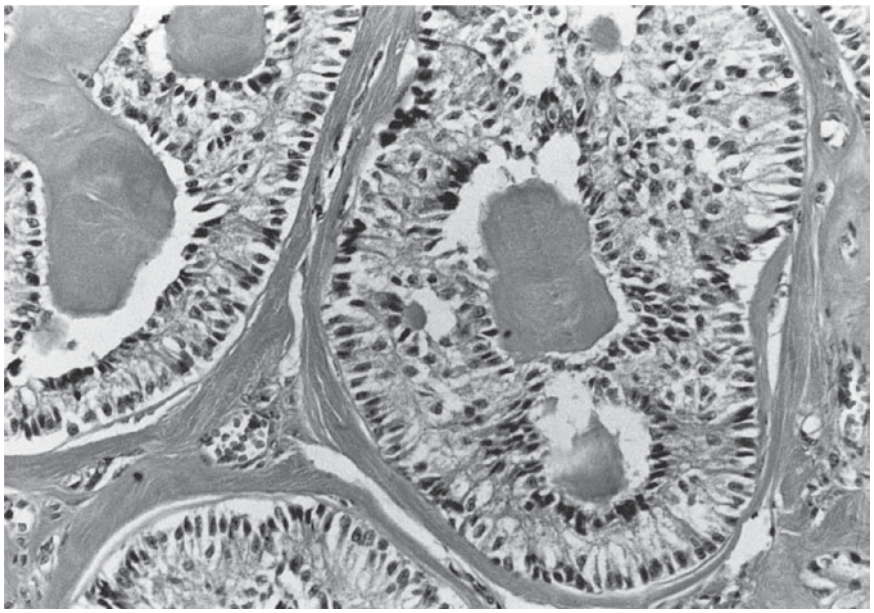

Fig. 14.25 Microscopic appearance of a sex cord tumor with annular tubules

presence of somatic mutations in this gene in sporadic sex cord-stromal tumors. However, the results obtained do not support a role for this gene in sporadic tumors [61]. In one study, loss of heterozygosity (LOH) at 19p13.3 was detected in 41% of sporadic sex cord-stromal tumors, but none showed somatic mutations or promoter hypermethylation of STK11 [62]. The authors hypothesize that LOH in that region targets a gene different from STK11. In this study, LOH at 19p13.3 was more common in granulosa and Sertoli-stromal cells (52%) than in the group of fibromas and thecomas (20%). Interestingly, the thecomas and fibromas that exhibited LOH contained occasional sex cord elements. In one study, LOH at 9q22.3, the region that contains the PTCH gene, was frequent in cellular fibromas and luteinized thecomas. In cellular fibromas, LOH at 9q22.3 occasionally coexisted with LOH at 19p13.3. The results are interesting since the PTCH gene mutations have been described in patients with Gorlin syndrome, which frequently exhibit ovarian fibromas. [63]

Karyotypic analyses of Sertoli–Leydig cell tumors have demonstrated the presence of trisomy 8 [64], isopchromosome 1q [65], trisomy 12 [66], and X chromosomal mosaicism [67, 68]. Comparative genomic hybridization (CGH) analysis [69] demonstrated the presence of amplification of a chromosomal region (+1q21.3–31) in one Sertoli cell tumor. In one study [70], using CGH and FISH, granulosa cell tumors were shown to exhibit more than one chromosomal abnormality. It was shown that sex cord-stromal tumors frequently exhibit a near diploid chromosome number, and 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. 14.26), by performing fluorescence in situ hybridization (FISH) with centromeric probes specific to chromosome 12 [66, 71, 72]. In one study [70], monosomy 22 was seen in 40% of granulosa cell tumors, while trisomy 12 occurred in 25%, monosomy X in 10%, and LOH at 17 in 5%. By CGH, gains of chromosome 12 and chromosome 25 were each detected in 33% of cases, LOH on chromosome 22 and X occurred in 35 and 5% of cases, respectively. In another study, chromosomal imbalances were seen in 61% of granulosa cell tumors, and involved frequently chromosomes 22, 14, and 12. [73]. In another study, monosomy 22 was suggested as a diagnostic aid in late recurrence of granulosa cell tumor [74]. Granulosa cell tumors also show epigenetic changes involving FHIT, FNACF, Cyclin D1, p16, ER-α, BRCA-1, RASSF1A, MGMT, CDH1, RAR-β, SYK, [75–77].

Finally, the neoplastic or reactive nature of the Leydig cell component in Sertoli–Leydig cell tumors has been addressed by molecular methods with contradictory results [78, 79].

Recently, one group of investigators have demonstrated the persence of a single, recurrent somatic mutation (402C–G) in FOXL2 in 97% of AGCT, 21% of thecomas and 10% JGCT. The mutation was absent in Sertoli-Leydig all tumor [80].

14.6 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,

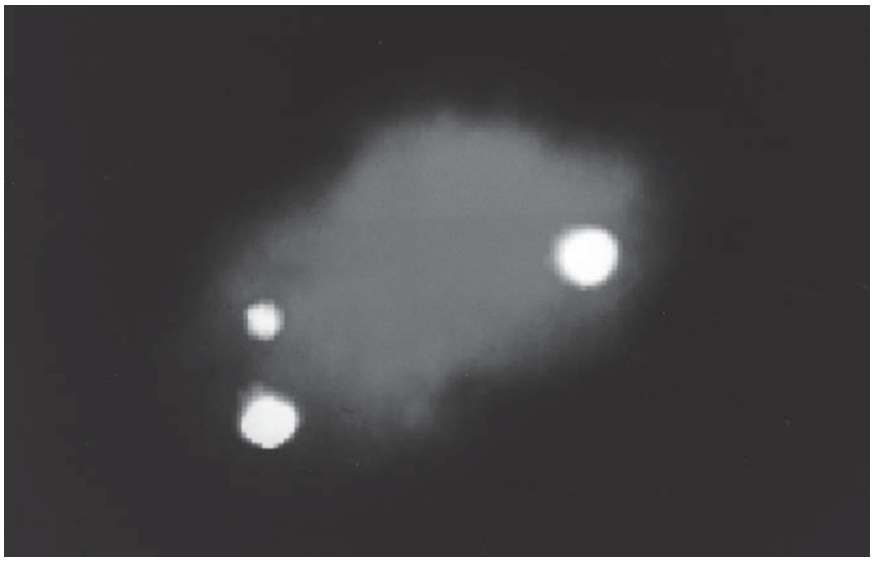

Fig. 14.26 Trisomy 12 detected by fluorescence in situ hybridization (FISH) with centromeric probes in isolated cells from a Sertoli-Leydig cell tumor

because they are frequently associated with hyperestrogenism or hirsutism and virilization [50].

Steroid cell tumors include three different tumor subtypes: Stromal luteoma, Leydig cell tumors, and steroid cell tumors NOS (not otherwise specified). From the immunohistochemical viewpoint, all steroid cell tumors have a common marker; all of them are positive for alpha-inhibin.

14.6.1 Stromal Luteoma

Stromal luteoma is a benign tumor, which accounts for 20% of steroid cell tumors, and usually occurs in post-menopausal 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. 14.27). Stromal luteoma usually develops in the context of pre-existing bilateral stromal hyperthecosis [81]. It is commonly associated with hyperestrogenism, although some infrequent cases have been associated with masculinization [82].

14.6.2 Leydig Cell Tumor

It is composed of Leydig cells. The vast majority of the reported tumors is located in the hilus and arises from hyperplastic hilus (Leydig) cells; they are named Hilus cell tumors. On rare occasions, Leydig cell tumors can occur within the ovarian stroma, far from the hilus; they are designated as Leydig (non-hilus) cell tumors.

Leydig cell tumors represent 20% of steroid cell tumors. They are benign, and usually occur in post-menopausal women. They are associated with various degrees of hyperandrogenism. Leydig cell tumors are small nodules, usually located in the ovarian hilus, and 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. 14.28a). 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 [83]. The tumors occasionally show fibrinoid material in the walls of medium-sized vessels (Fig. 14.28b)

14.6.3 Steroid Cell Tumors (NOS, Not Otherwise Specified)

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. It represents 60% of steroid cell tumors. In one series, clinically malignant tumors accounted for 25% of the cases [84].

Steroid cell tumors NOS may show different gross and microscopical features. Some tumors are brown in color while others are yellow (Fig. 14.29). Some tumors are composed of polygonal-shaped eosinophilic cells with lypochrome, whereas others contain large cells with clear cytoplasms and abundant lipids.

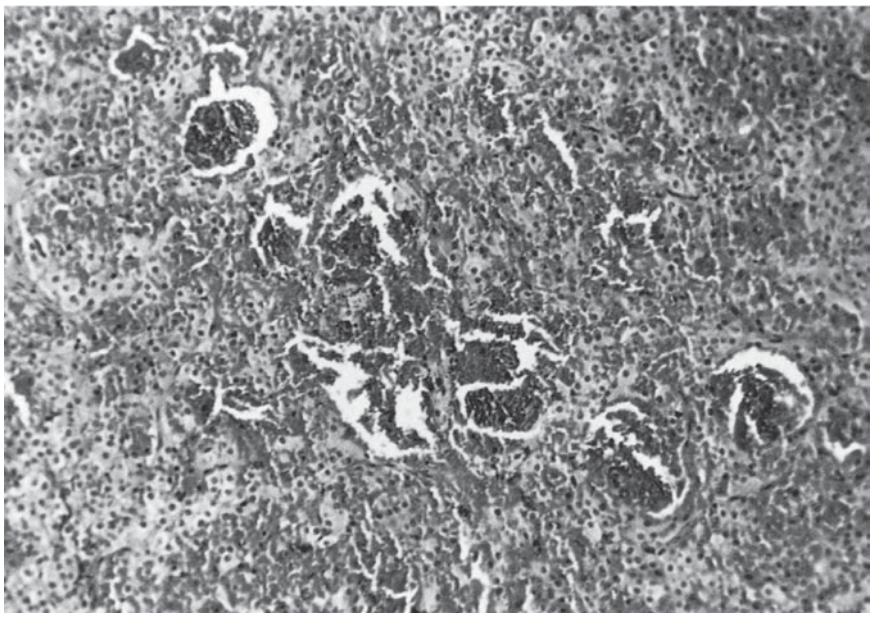

Fig. 14.27 Irregular spaces containing blood in a stromal luteoma

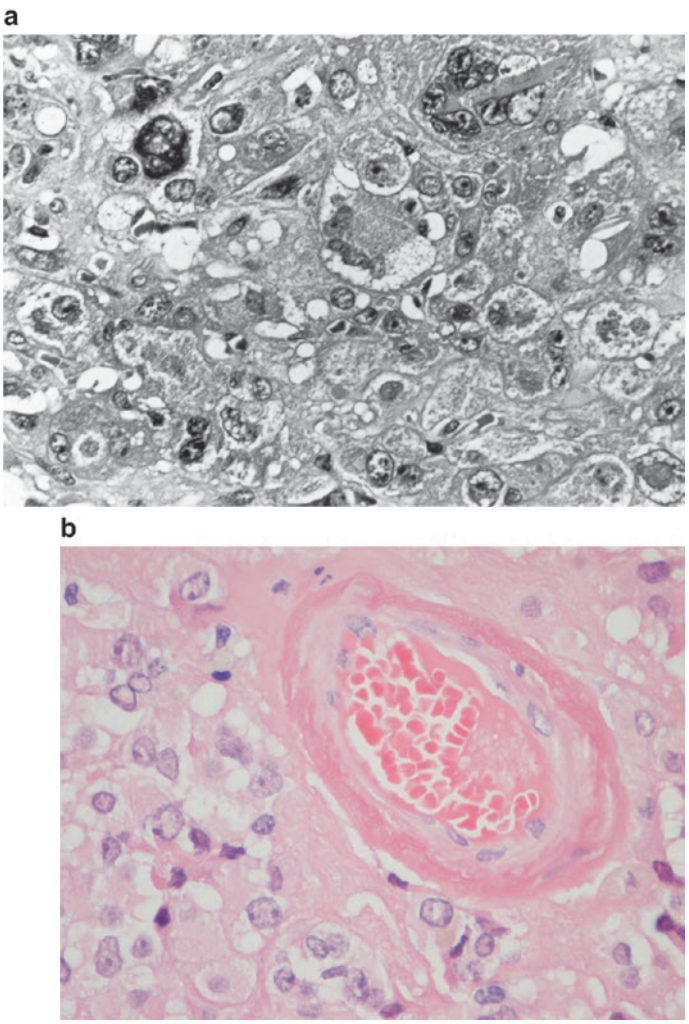

Fig. 14.28 (**a**) Marked nuclear pleomorphism and occasional Reinke crystalloids in an ovarian hilus cell tumor. (**b**) Fibrinord material in the walls of blood vessels

Fig. 14.29 Gross appearance of a steroid cell tumor, NOS

There are several clinicopathologic criteria to be taken into account when considering the possibility of malignancy. Age of presentation is a good indicator or malignant behavior; since the vast majority of malignant tumors occur in women older than 51 years. Other features to be considered are: (1) more than 2 mitosis per 10 hPF; (2) size over 7 cm; (3) hemorrhage, and (4) the presence of marked nuclear atypia.

Some examples of steroid cell tumors, NOS, are tumors originating from ectopic adrenal cortical rest, and may be associated with Cushing's Syndrome.

14.6.4 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 are some evidences suggesting a role for activating mutations of the Luteinizing Hormone Receptor (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 [85, 86]. 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 [87]. The mutation consisted in the substitution of cytosine for guanine at nucleotide 1732, which encodes 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 [88, 89].

A different group of investigators identified activating mutations in the α subunit of G_s in three ovarian and one testicular Leydig cell tumors [90]. 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 Gα 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 Gα subunit. Mutations of the α-subunit genes that lead to their 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 [91].

Steroid cell tumors are usually androgenic, but they can be associated with the production of renin, and can also be responsible for the secondary polycythaemia [92]. Figure 14.30 shows the results of RT-PCR in the detection of renin in a steroid cell tumor that was associated with hypertension and secondary polycythaemia.

14.7 Germ-Cell Tumors and Gonadal Dysgenesis

14.7.1 Germ Cell Tumors

Ovarian germ cell tumors represent 30% of all ovarian tumors. Over 95% of them 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%) shows 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

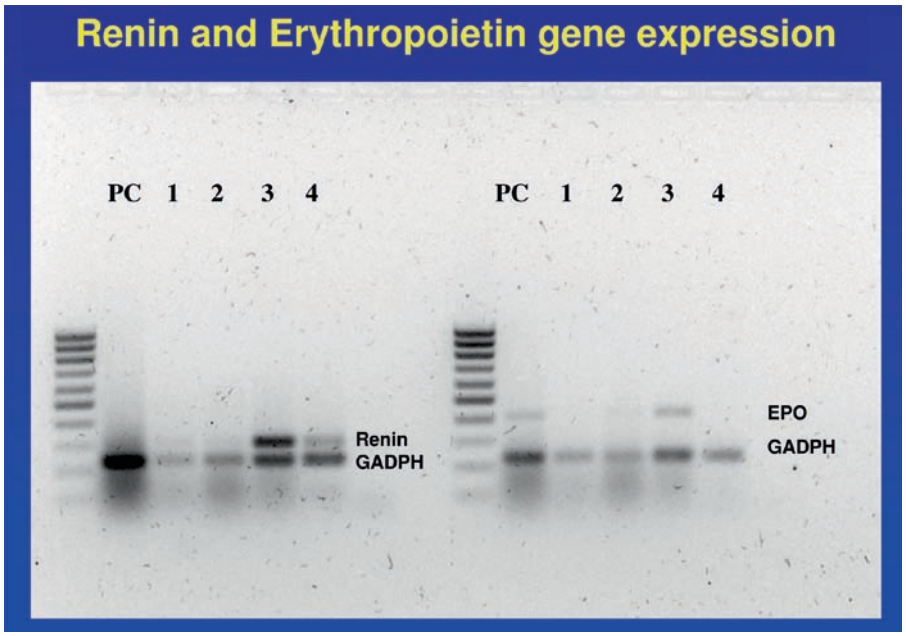

Fig. 14.30 RT-PCR analysis in a steroid cell tumor that was associated with hypertension and polycythemia. RT-PCR demonstrate the presence of renin mRNA, and absence of erythropoietin (EPO) in tumor cells. (Lanes PC, 1, 2 and 3 correspond to several different positive and negative controls. Lane 4 corresponds to tumor RNA).

discoloration. It shows 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 below, dysgerminoma may develop from a pre-existing 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 years. It may show a wide variety of microscopic patterns, and may exhibit foci of hepatic and enteric differentiation. It is usually associated with elevated α-Fetoprotein serum levels. Embrional 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. 14.31), which may be functioning, and even be associated with tyrotoxicosis. Similarly, monodermal teratomas may be exclusively composed of neuroendocrine cells with 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. 14.32). 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 arises 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.

14.7.2 Gonadal Dysgenesis

As mentioned before, gonadoblastoma and dysgerminoma may arise from gonadal tissue in phenotypic females with pure or mixed gonadal dysgenesis [93, 94] (Fig. 14.33). 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

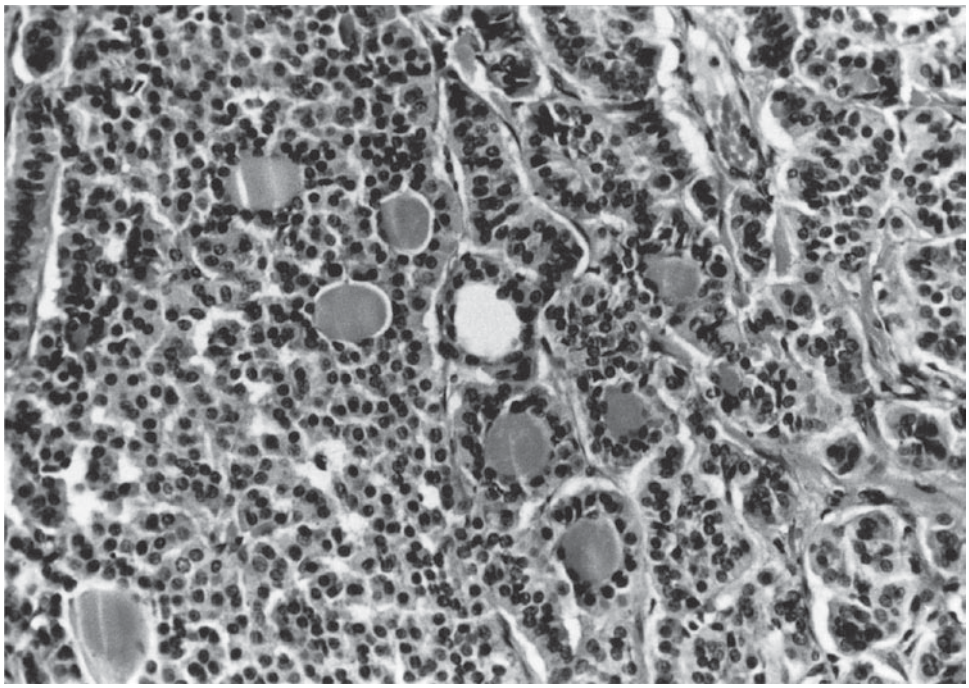

Fig. 14.31 Strumal carcinoid combines the presence of struma ovarii and a neuroendocrine (carcinoid) component

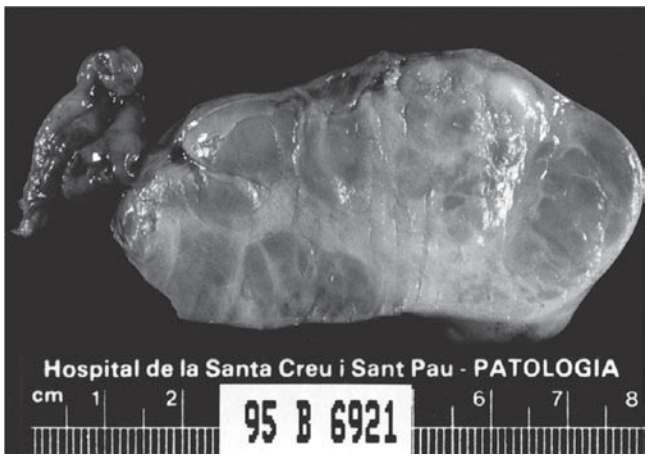

Fig. 14.32 Gross appearance of a "burn out" dysgerminoma in a gonadal streak from a patient with mixed gonadal dysgenesis

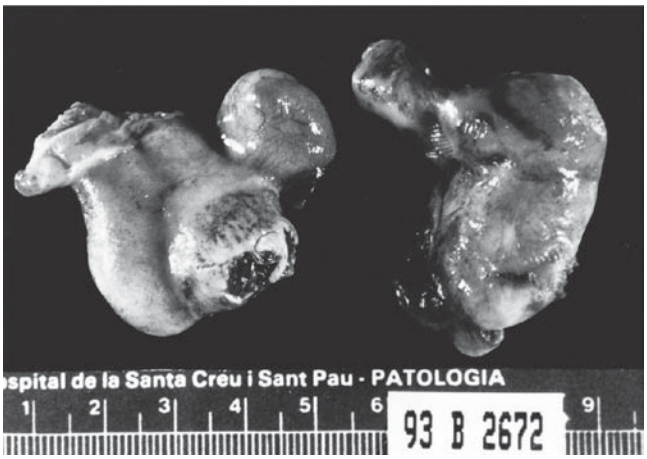

Fig. 14.33 Gross appearance of the abdominal gonads removed from a patient with androgen insensitivity syndrome

and may be responsible for some androgenic manifestations as well as for gradients in testosterone and delta-4-androstendione concentrations in gonadal blood obtained during surgery.

14.7.3 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. 14.34). 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 [94]. 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 and malignant germ cell tumors are also sometimes detected [95].

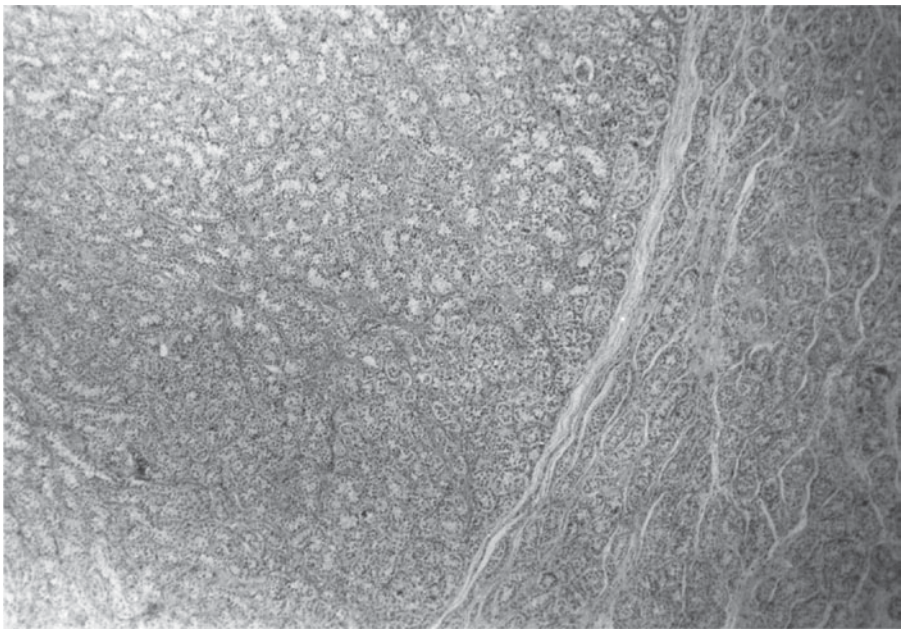

Fig. 14.34 Microscopic appearance of a so-called Sertoli cell adenoma in the gonad from a patient with androgen insensitivity syndrome

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Chapter 15
Endocrine Testis

Robert E. Emerson, Liang Cheng, and Thomas M. Ulbright

15.1 Introduction

Each major histologic category of testicular tumor includes hormonally functional neoplasms (Table 15.1). Testicular germ cell tumors, sex cord-stromal tumors, and tumors of neuroendocrine origin are all 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 serve 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. Proteins synthesized by neoplastic cells, such as gonadotropins, may stimulate secretion of androgens and estrogens from non-neoplastic testicular cells. Finally, hormones produced at other sites may be altered within tumor cells, possibly enhancing their effect. Additionally, hormones produced at other sites may induce tumor-like hyperplasias in the testis that contribute to secondary hormone manifestations.

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, will also be considered in detail. These tumors are derived from endocrine cells and, consequently, much more frequently demonstrate hormone production. Leydig cell hyperplasia will be discussed, as it may histologically simulate neoplasia. Finally, tumors believed to originate from neuroendocrine cells within the testis are considered.

15.2 Germ Cell Tumors

Germ cell tumors comprise the vast majority of primary testicular neoplasms. Preexisting endocrine dysregulations such as hypoandrogenism, prenatal hyperestrogenism, and increased circulating gonadotropin levels may predispose to testicular germ cell neoplasia [4, 5]. The incidence of testicular germ cell neoplasms has been increasing over the past several decades [6] in association with a decline in mean sperm count and it is possible that the two are related [7]. Testicular germ cell tumors arise from intratubular germ cell neoplasia, unclassified (IGCNU or testicular "carcinoma in situ"). The cells of IGCNU morphologically and in terms of gene expression resemble fetal germ cells and it has been proposed that IGCNU may, in part, be a consequence of arrested development of fetal germ cells, in some cases resulting from environmental endocrine disruptors [8–10]. This arrest of development is thought to result in a testicular dysgenesis syndrome [11–14]. Direct relevance of this syndrome to patient care includes the reduced spermatogenesis seen in the contralateral testis of men with germ cell tumors relative to normal controls [15] and the presence of IGCNU in the contralateral testis of 5% of patients with testicular germ cell tumors [16]. Testicular endocrine function is impaired in germ cell tumor patients, and irradiation of the contralateral testis if IGCNU is discovered on biopsy may further decrease Leydig cell function [16].While it is believed that prenatal exposure to environmental estrogenic compounds may relate to the increasing incidence of testicular carcinoma, others have argued that there is, as yet, little direct evidence for this assumption [17].

The morphologic and immunohistochemical approach to the diagnosis of testicular germ cell tumors continues to be a subject of interest, due to the challenge of differentiating some tumor types and the availability of several new

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Table 15.1 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
Intratubular large cell hyalinizing Sertoli cell neoplasia
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

immunohistochemical markers [18–21]. The morphologic diagnosis of seminoma is usually straightforward, but *can be* complicated by variants that include a microcystic or tubular architecture [22], plasmacytoid morphology [23], signet ring cell change [24], and exclusive intertubular growth [25]. The tumor is histologically usually solid in architecture with cells with moderate-sized polygonal nuclei and a moderate amount of pale-staining to clear cytoplasm. Lymphocyte-rich fibrous bands in association with the neoplastic cells are a characteristic feature. Seminomas are generally positive with immunohistochemical stains for placental alkaline phosphatase, typically show only weak and focal staining with keratin stains, and are negative for CD30, AFP, and inhibin [26–30]. Newer, highly sensitive markers for seminoma include OCT4 [31], NANOG [32], podoplanin [33], and SOX17 [34], although OCT4 and NANOG are also positive in embryonal carcinoma.

Embryonal carcinomas may have glandular, papillary, solid, or mixed architecture and are composed of polygonal cells with moderately abundant amphophilic cytoplasm. The nuclei are typically irregular and may have chromatin clumping and a paritally clear or vesicular appearance. Embryonal carcinomas typically stain for placental alkaline phosphatase, CD30, NANOG, SOX2, and OCT4 [28, 30–32, 34–36].

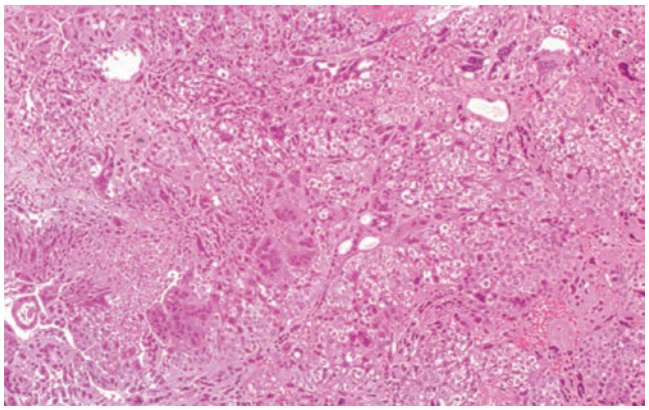

Fig. 15.1 While pure choriocarcinoma is rare, it is a common component of mixed germ cell tumors and can be recognized by the presence of cytotrophoblast cells in association with syncytiotrophoblast cells

Yolk sac tumors have a highly variable histologic appearance with several potentially confusing patterns such as the solid pattern, the microcystic pattern, and hepatoid yolk sac tumor. They are typically strongly positive for keratin AE1/AE3, while AFP staining is variable, and OCT4 is negative [31, 37]. Glypican 3 appears to be more sensitive than AFP as a marker for yolk sac tumor [38].

Choriocarcinoma is composed of cytotrophoblast and syncytiotrophoblast cells (Fig. 15.1) and, as expected, consistently stains for hCG. It should be noted that seminoma and other germ cell tumor types may have associated syncytiotrophoblast cells that also mark with this stain.

The most consistent cytogenetic finding in testicular germ cell tumors is over-representation 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 [39]. The majority lack significant endocrine manifestations. Many germ cell tumors, especially non-seminomatous 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.

Clinically, the most important serum tumor markers are hCG and alpha-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 [40]. Serum AFP is produced consistently by yolk sac tumor elements, and the degree of elevation in mixed germ cell tumors correlates with the amount of yolk sac tumor present [41]. 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.

TRA-1-60 has been studied as a specific marker for embryonal carcinoma [42].

Seminoma generally lacks significant endocrine manifestations [3]. Human chorionic gonadotropin (hCG) is, however, readily demonstrated by immunohistochemistry in the syncytiotrophoblast cells commonly encountered within seminomas (Fig. 15.2) 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 [43]. Occasionally, patients with seminoma with syncytiotrophoblast cells have gynecomastia secondary to peripheral conversion by aromatase of androgens secreted from hCG-stimulated Leydig cells to estrogens [44]. In a single reported case, gynecomastia was thought to be associated with a pure seminoma that produced estradiol and estradiol production was confirmed following excision in the laboratory [45]. Hyperandrogenism caused by seminomas with syncytiotrophoblast cells may result in secondary polycythemia [46, 47].

Rarely, hypercalcemia due to production of a presumed parathyroid hormone-like substance by a seminoma is seen [48, 49]. Resolution of hypercalcemia may occur with orchiectomy and retroperitoneal radiotherapy. Paraneoplastic autoimmune reactions associated with seminoma include limbic and brainstem encephalopathy [50–52], hemolytic anemia [53], exophthalmos [54, 55], and membranous glomerulonephritis [56]. 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 [57]. 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 [58].

Choriocarcinoma may be associated with several clinical endocrine syndromes. Gynecomastia is the most common, affecting 10–20% of patients [3]. Production of hCG results in Leydig cell hyperplasia because of its luteinizing hormone-like activity. The increased androgens then undergo peripheral conversion in adipose tissue to estrogens by aromatase [59]. The indirect production of estrogens by this mechanism has permitted estradiol elevation to be used as an indicator of tumor recurrence in both seminoma and non-seminomatous germ cell tumors [60]. 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 [61]. The mechanism of hyperprolactinemia is unclear, but may result from estrogen elevation or stimulation of pituitary lactotrope cells by the free alpha subunit of hCG [61]. 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 trophoblastic cells [3]. It is also well known that pure teratoma of the testis may have non-teratomatous metastases, including choriocarcinoma, which could be responsible for this occurrence.

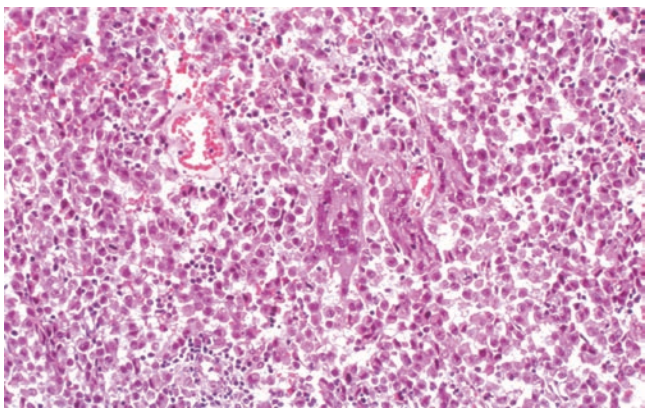

Fig. 15.2 Pure seminoma commonly has occasional syncytiotrophoblast cells, which are not by themselves diagnostic of choriocarcinoma

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 [62, 63].

15.3 Sex Cord-Stromal Tumors

15.3.1 Leydig Cell Tumors

Normal Leydig cells are mesenchymal in origin and are arranged as single cells and in small clusters in the generally loose connective tissue 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 [64].

Leydig cells are the primary site of testicular steroid synthesis. Leydig cells respond to luteinizing hormone (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 [65].

Leydig cell tumors comprise 1–3% of primary testicular tumors and are seen in both boys and adult men [65]. In one series, the average age at presentation was 47 years with a range of 2–90 years [66]. In patients with Leydig cell tumors,

many endocrinologic alterations have been noted [67–69], and isosexual pseudoprecocity resulting from a Leydig cell tumor has been reported in boys as young as 1 year of age [70]. 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 [67].

Some Leydig cell tumors in children are associated with a somatic, heterozygous activating mutation in the gene for the luteinizing hormone receptor [71–75]. Such mutations are not identified in other cases of pediatric Leydig cell tumor [76]. Uncommon Leydig cell tumors in adults may harbor a similar mutation or be associated with a germline mutation in the fumarate hydratase gene, a genetic change that is seen in patients who have hereditary leiomyomatosis and renal cell cancer syndrome [77].

Hormonal manifestations, including precocious pseudopuberty and gynecomastia, are usually observed in children with Leydig cell tumors [3, 78]. Leydig cell tumors in both children [76] and adults [79] may present with hormonal effects in the absence of a palpable mass and in this setting serum and urine androgen elevation, testicular ultrasound, and selective venous sampling can provide the diagnosis [80]. 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 [66, 81], although approximately 15% of adults present because of gynecomastia [66]. 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 [81]. 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 [82]. 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 [83]. Regression of gynecomastia following orchiectomy is the usual outcome [68, 69, 80, 84]. Endocrine testicular function and spermatogenesis may, however, be impaired on a long-term basis in the contralateral testis [84, 85].

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 are typically yellow or yellow-brown [66]. The nodules may be divided by white fibrous bands.

The cells of a Leydig cell tumor are most commonly arranged diffusely, although trabeculae, cords, and tubule-like structures may also be seen. They are typically large and polygonal with eosinophilic, granular cytoplasm (Fig. 15.3). Reinke crystals are seen in 35% of cases on careful examination [66]. Adipose differentiation, ossification, and spindle cell areas may be seen [86]. Rarely, diffuse spindle cell or frankly sarcomatoid differentiation may occur [86, 87]. A microcystic pattern is also unusual and may be confused with yolk sac tumor [88]. Distinguishing the two is critical for clinical management as patients with yolk sac tumor typically receive chemotherapy while those with Leydig cell tumors are either followed or have retroperitoneal lymphadenectomy. Negative AFP and placenta-like alkaline phosphatase and positive reactions for inhibin-alpha and Melan A (A103) are expected in Leydig cell tumors in contrast to yolk sac tumors.

The surrounding testis typically shows decreased spermatogenesis and atrophy of surrounding Leydig cells [69]. Although progressive degeneration of normal Leydig cells surrounding a Leydig cell tumor is probably the typical finding [89], Leydig cell hyperplasia may occasionally be seen [68].

Ultrastructurally, "membranous whorls" have been observed in Leydig cell tumors but not in normal Leydig cells [67]. By immunohistochemistry, Leydig cell tumors are usually positive for vimentin and inhibin while cytokeratin, S-100 protein, epithelial membrane antigen, and desmin are less consistently expressed [90, 91]. Immunohistochemical staining for A103, an antibody to the Melan-A antigen, is positive in Leydig cell tumors [92]. 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 [65] and testis-sparing surgery has occasionally been performed [78]. In adults, approximately 10% behave in a malignant

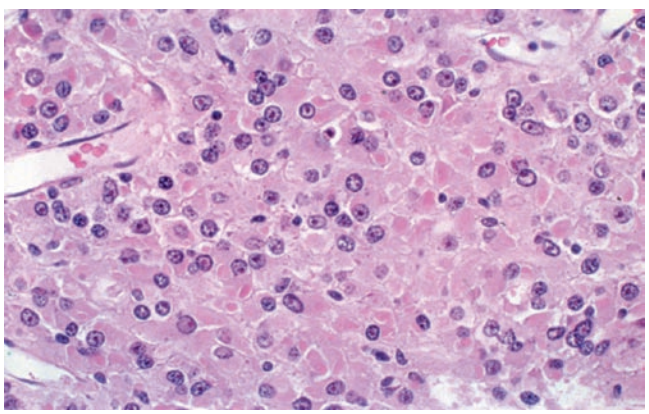

Fig. 15.3 Leydig cell tumors are composed of sheets of polygonal cells that displace and compress the surrounding seminiferous tubules. The nuclei are round, typically with a single prominent nucleolus, and the cytoplasm is eosinophilic. Cytoplasmic Reinke crystals are seen in some cases, with this case showing globular variants of Reinke crystals

manner with retroperitoneal lymph node, pulmonary, and liver metastases being most common [93, 94]. 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 [66, 95, 96]. 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 [95, 96]. The presence or absence of these features, however, neither guarantees nor excludes the possibility of metastasis [93] and there is no clear evidence that these features have relevance in pediatric cases. Therefore, benign Leydig cell tumors can only be identified unequivocally by the absence of metastatic tumor on prolonged follow-up, but the features mentioned above 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 represent a form of steroid cell hyperplasia having Leydig cell-like morphology, as adrenal rests are not believed to occur within the testis itself [97]. 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 [64]. Although serum ACTH and 17-hydroxyprogesterone elevation are expected in the usual form of the adrenogenital syndrome, one case of Leydig cell tumor which produced both of these hormones has been reported [98]. 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 steroid cell nodules in the adrenogenital syndrome contrast with the solitary, unilateral nature of Leydig cell tumor. Additionally, the tumors of the adrenogenital syndrome often have a prominent and distinctive fibrous tissue component that tends to compartmentalize the lesional cells into fairly discrete groups. Furthermore, most lesions of adrenogenital syndrome 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. In contrast to Leydig cell tumor, cytoplasmic Reinke crystals are not observed.

Inguinal orchiectomy is the treatment of choice in adult men with suspected Leydig cell tumor, although there have been good results in selected patients with small tumors who underwent local excision and whose tumors had favorable pathologic features. In children with suspected Leydig cell tumor, testis-sparing surgery with enucleation of the mass is an alternative to orchiectomy [65]. Retroperitoneal lymphadenectomy is the *mainstay* of treatment for malignant examples.

15.3.2 Leydig Cell Hyperplasia

Leydig cell hyperplasia (Fig. 15.4) may be seen in several clinical settings including tuberculosis, syphilis, carcinoma, pernicious anemia, alcoholism, chronic spermatic cord compression, chronic disease of the bladder and prostate, Klinefelter syndrome, and myotonic dystrophy, type 1 [64, 99]. Congenital Leydig cell hyperplasia may be observed in infants of diabetic mothers, Rh incompatibility, and Beckwith-Wiedemann syndrome [100]. It may also be idiopathic and familial [101], with premature Leydig cell proliferation resulting in male-limited familial pseudoprecocity [102]. This syndrome is gonadotropin independent, unlike the more common central precocious puberty [102, 103]. Small Leydig cell nodules often develop in male-limited familial pseudoprecocity [104].

Hyperplasia of steroid cells resembling Leydig cells may be seen in the adrenogenital syndrome, and some cases previously reported as bilateral Leydig cell tumor of childhood likely are hyperplastic in nature [3]. The presence of bilateral testis masses composed of Leydig-like 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 [65].

Occasionally, Leydig cell hyperplasia may be seen in the interstitium surrounding a Leydig cell tumor [68]. This phenomenon may be explained by production of a stimulating factor by the tumor [68] or by the neoplastic transformation of the cells of preexisting Leydig cell hyperplasia [64]. Leydig cell hyperplasia may also accompany hCG-producing testicular tumors [43]. In these cases, large but

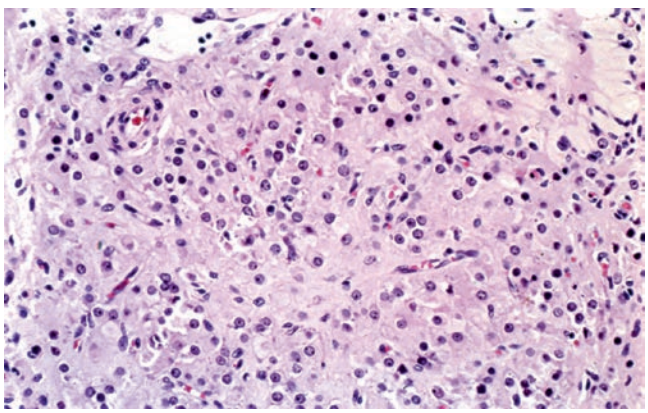

Fig. 15.4 Leydig cell hyperplasia

discontinuous clusters of Leydig cells may be seen surrounding a germ cell tumor that contains syncytiotrophoblast cells.

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 [105]. They have the typical cytological features and usually occur as small nests in close association with nerve fibers.

15.3.3 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 [106, 107]. The adrenogenital syndrome results most commonly from 21-alpha-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 or partial deficiencies may result in milder and often later presenting forms in which a testis mass is occasionally the presenting finding [65]. 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 [108]. Two-thirds of patients with testicular tumors of the adrenogenital syndrome have a salt-losing adrenal disorder [108]. These tumors present at an average age of 23 years and 83% are bilateral [108]. Androgen excess, in one case resulting in aggressive behavior, has been observed due to these tumors [109]. In one ultrasound study, 16 of 17 patients with adrenogenital syndrome had testicular tumors ranging in size from 0.2 to 4.0 cm [110]. One case was associated with cryptorchidism, seminoma, and testicular myelolipoma [111]. One case of an 8.5 cm malignant Leydig cell tumor arising in the adrenogenital syndrome has been reported [112].

Testicular tumors submitted for pathologic evaluation in the adrenogenital syndrome are usually greater than 2 cm, solid, unencapsulated, divided into nodules by fibrous bands, and green-brown in color [108]. They may histologically closely resemble Leydig cell tumors, and can be mistaken for such, possibly resulting in unnecessary orchiectomy [113–115]. The tumor cells are arranged in nests divided by fibrous stroma (Fig. 15.5). The cells have abundant eosinophilic cytoplasm with lipochrome pigment but lack Reinke crystals [108]. In the lipoid congenital adrenal hyperplasia variant, lipid vacuole-containing steroid 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 [116].

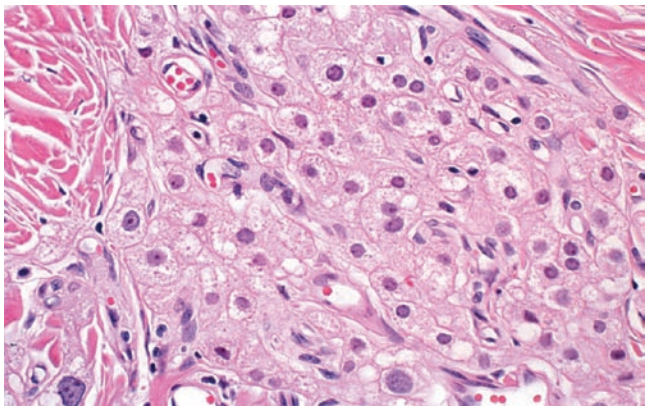

Fig. 15.5 The cells of the testicular tumor of the adrenogenital syndrome closely resemble those of Leydig cell tumor. The history of endocrine abnormalities, bilaterality, and multifocal distribution of tumor is critical for the recognition of this tumor

The cell of origin of these "tumors" has been disputed [108]. 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 [97]. Against Leydig cell origin are the facts that Reinke crystals are not identified in them [108] and that the tumors develop in a state of ACTH excess and biochemically mimic the function of adrenal cortex [65]. Origin from pluripotential hilus cells has been proposed [108].

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, as are the prominent fibrous bands seen on low power examination. Orchiectomy should not be performed in boys with precocious pseudopuberty and a testicular tumor without a complete endocrinologic evaluation, which rules out the adrenogenital syndrome [117]. These tumors do *not* require surgical treatment, with regression occurring with steroid replacement therapy in many cases [65, 118], an observation supporting that they are non-neoplastic nodular hyperplasias.

15.3.4 Sertoli Cell Tumors

Sertoli cell tumors have been thought to comprise approximately 0.5% of adult and 9.0% of childhood testicular tumors [119, 120], but a more recent study found that they account for only 1.3% of pediatric testis tumors [121]. Some cases reported as Sertoli cell tumors may, in fact, represent

hyperplastic Sertoli cell nodules. Sertoli cell nodules with production of associated basement membrane-like hyaline material are especially common in undescended testes [122]. Gynecomastia and impairment of libido have been reported in association with Sertoli cell tumors. Boys with Sertoli cell tumors may present with gynecomastia in the absence of associated virilizing features. This contrasts with the situation with pediatric Leydig cell tumors where boys with gynecomastia invariably also have virilization. The molecular mechanisms responsible for Sertoli cell tumor development are poorly understood, but gains of X chromosomes are a common cytogenetic abnormality [123]. Mice with inactive genes for inhibin-alpha develop Sertoli cell tumors with 100% penetrance [124].

Sertoli cell tumors grossly are well-circumscribed, gray to yellow-white, and solid, sometimes with cystic areas [119, 125]. Microscopically, solid areas and tubular structures usually are apparent (Fig. 15.6). The amount of tubular differentiation is variable. Retiform structures may be seen in some cases. The tumor cells have a moderate amount of eosinophilic to clear cytoplasm [125]. Some cases may display extensive sclerosis, and these may be assigned to a specific subtype, sclerosing Sertoli cell tumor [126, 127]. Sarcomatoid differentiation with osteosarcoma-like foci has been reported in one case [128]. Some malignant cases have a mainly diffuse arrangement of cells with clear cytoplasm and lymphocytic infiltrates that resembles seminoma (Fig. 15.7), although the lower grade nuclear morphology and mitotic rate, as well as immunohistochemical staining results help distinguish the two [129].

Sertoli cell tumors are positive for vimentin and, in some cases, for keratin, epithelial membrane antigen, and smooth muscle actin as well [120, 129]. Inhibin and calretinin mark sex cord-stromal tumors, including Sertoli cell tumors [91], but while many Sertoli cell tumors are inhibin positive, only a minority of malignant cases are inhibin positive [130]. S-100 protein immunostaining may also be seen in some cases [90]. Placental alkaline phosphatase is negative, in contrast to seminoma, which also shows negative reactivities for epithelial membrane antigen, inhibin-alpha, and AE1/AE3 cytokeratin. Chromogranin A and synaptophysin may be positive in Sertoli cell tumors [131].

Although the majority of adult Sertoli cell tumors are benign, approximately 10% have been associated with metastases [119]. Malignant Sertoli cell tumors may histologically mimic seminoma and in this setting immunohistochemical staining for placental alkaline phosphatase, OCT4, and inhibin is particularly useful [129]. In 18 cases of pediatric Sertoli cell tumors, no recurrences were observed following orchiectomy [120], 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.

15.3.5 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 [132]. Less commonly, testosterone production may be observed [133]. 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 [134]. Later, it was determined that these associations were due to their occurrence as a part of Carney complex.

While large cell calcifying Sertoli cell tumor is characteristic of Carney complex [135], it may also be seen in patients who are *not* affected by this disorder [136]. Those cases not associated with Carney complex usually present between 13 and 34 years of age, are more likely to be unilateral and

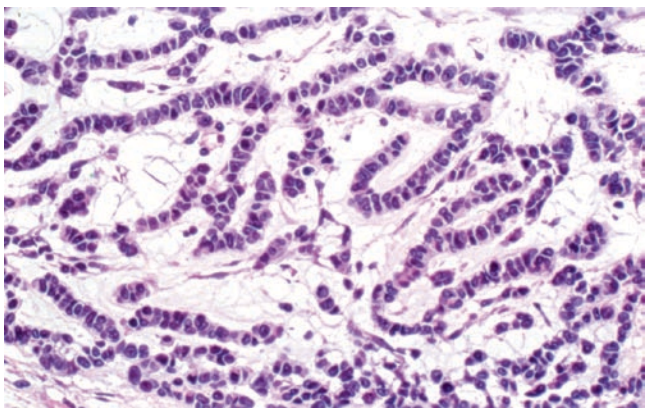

Fig. 15.6 Trabecular architecture is a frequent finding in Sertoli cell tumor

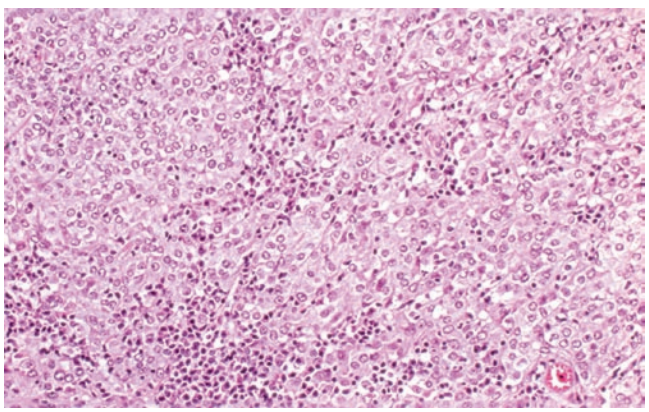

Fig. 15.7 This malignant Sertoli cell tumor has solid architecture, clear cytoplasm, and a lymphocytic infiltrate. Tumors such as this one may be confused with seminoma

unifocal, and can be treated with orchiectomy alone or even local excision [136–138], unless features associated with malignant behavior are identified [139]. Rare fatal cases of malignant large cell calcifying Sertoli cell tumor have been reported [140]. One case of large cell calcifying Sertoli cell tumor was associated with unilateral renal agenesis and inferior vena cava duplication, but this may be only a chance association [137].

Grossly, the tumors average approximately 2 cm at presentation and are well circumscribed, solid, and yellow- to gray-tan [132, 136]. 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 [136]. The tumor cells are large with lightly to densely eosinophilic cytoplasm [132]. Centrally located small spherical or larger amorphous calcifications are typical but not always present (Fig. 15.8) [136]. An interesting finding in some cases is the presence of alpha-1-antitrypsin-containing cytoplasmic hyaline globules in Sertoli cells in the uninvolved seminiferous tubules [136].

Charcot-Böttcher crystalloids, unique to Sertoli cells, may be seen by electron microscopy as ellipsoidal aggregates of filaments [132, 133]. Tumor nests are surrounded by a basal lamina [141]. Cytoplasmic dense granules, lipid droplets, and intracytoplasmic lumina with microvilli may be seen [141]. Strong vimentin and focal keratin staining has been noted by immunohistochemistry [136]. Staining for S-100 protein, inhibin-alpha, calretinin, melan-A, and CD10 may be positive [141, 142]. Inhibin may be useful as a serum tumor marker [143].

These tumors generally behave in a benign fashion [138], however, occasional cases are malignant [139, 144, 145]. Malignant tumors may have endocrine manifestations including gynecomastia [145]. 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 years versus 17 years), 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 over 4 cm, and were microscopically more likely to show necrosis, cellular atypia, high mitotic rate (greater than 3 per 10 high power fields), and vascular invasion [139].

15.3.6 Granulosa Cell Tumor

As in the ovary, two types of granulosa cell tumor are recognized: adult type and juvenile type. Granulosa cell tumors of adult type very rarely occur in the testis. They 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 [146]. The adult type has been reported in men from 16 to 76 years of age [147] and may be associated with hyperestrogenemia and resulting gynecomastia and loss of libido [3]. However, these tumors more commonly lack endocrine manifestations [147].

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, rosette-like structures with a round, smooth-bordered central space that often contains lightly eosinophilic secretion, are seen in about half of the cases (Fig. 15.9). The tumor cells have scant cytoplasm and oval, pale staining nuclei. A *key* cytologic finding is the presence of longitudinal nuclear grooves [147]. The tumor cells are immunohistochemically positive for vimentin and negative for epithelial membrane antigen [147]. Focal cytokeratin positivity has been reported [148], but not in all studies [147, 149]. Membranous O13 (CD99, Ewing sarcoma antibody)

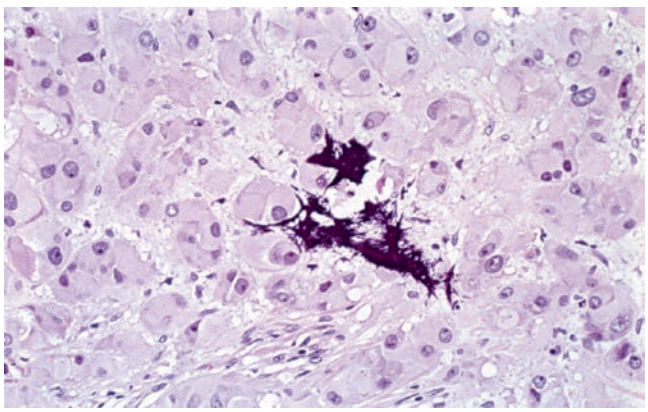

Fig. 15.8 Cells with abundant eosinophilic cytoplasm and foci of calcification characterize large cell calcifying Sertoli cell tumor

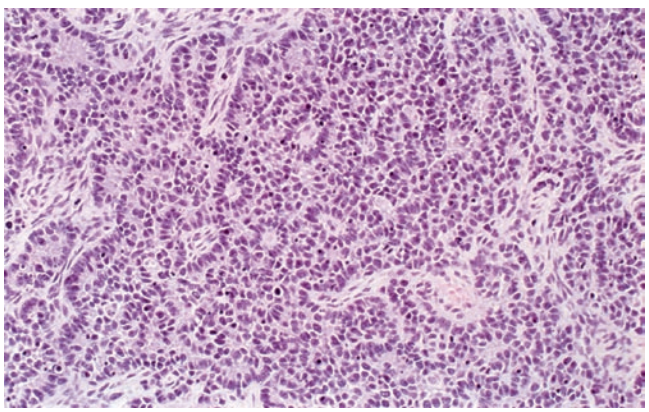

Fig. 15.9 Adult-type granulosa cell tumor is composed of sheets of cells with pale cytoplasm and rosette-like Call-Exner bodies. At higher power longitudinal nuclear grooves are seen in the oval nuclei

positivity has also been noted [148]. Ultrastructural findings include occasional desmosomes, cytoplasmic glycogen, rough endoplasmic reticulum, and folded nuclear membranes, similar to ovarian granulosa cell tumors [150].

Retroperitoneal or other metastases have been documented in 4 of 19 cases (21%) reported or reviewed in one study [147]. Clinical and histologic findings do not reliably predict behavior in this group of tumors, but large size (greater than 7 cm), lymphovascular invasion, and necrosis are seen more frequently in tumors that metastasize [147]. In one case, survival for 14 years with no recurrence followed orchiectomy, retroperitoneal lymphadenectomy, and radiation therapy in a patient with retroperitoneal lymph node metastases [151].

Juvenile granulosa cell tumors may be *congenital* or they may present in infants, usually at less than 6 months of age [152]. Rare cases may be seen in older boys up to 4 years of age [153]. They account for, approximately, 6% of childhood testicular tumors [120]. They may be associated with testicular torsion [154]. In some cases, they are associated with ambiguous external genitalia, an abnormal somatic karyotype, or mixed gonadal dysgenesis [155, 156]. 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 [120]. Occasionally, extensive cystic change may be seen [156]. Histologically, they are composed of cells arranged in lobules and that focally form follicle-like structures with central lumens having mucin-containing fluid and a lining of several layers of cells (Fig. 15.10). 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 [29, 120, 157]. Ultrastructurally, muscle-like filaments with dense bodies are apparent, suggesting dual epithelial and mesenchymal differentiation [157]. Aberrant expression of FOXL2, a gene involved in differentiation of the early undifferentiated gonad into the ovary, has been demonstrated by immunohistochemistry in testicular juvenile granulosa cell tumors [158].

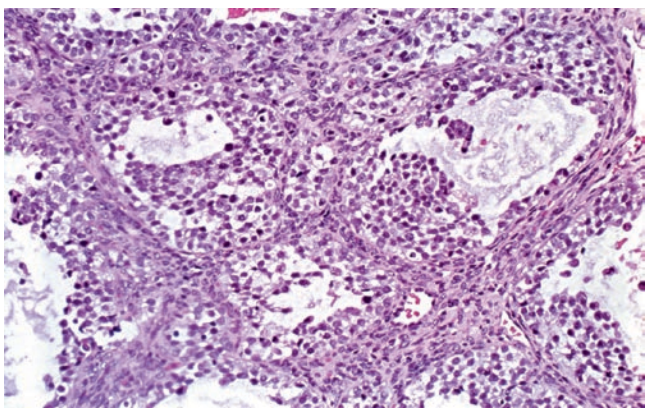

Fig. 15.10 In juvenile-type granulosa cell tumor, follicle-like structures or cystic change may be seen. The nuclear grooves typical of adult-type granulosa cell tumor are not typically seen

In 3 series totaling 32 cases, *no* recurrences were noted following orchiectomy [120, 152, 157]. More recently, testis-sparing surgery has been recommended [159].

15.3.1 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 [160–164]. Actin positivity in some cases suggests origin from peritubular myoid cells [161, 162, 164]. While these tumors may have morphologic and immunohistochemical features suggesting differentiation into both Leydig and granulosa cells [165], endocrinologic manifestations have not been reported and these tumors will not be considered further here.

15.4 Neuroendocrine Tumors

15.4.1 Ectopic Endocrine Tissue

Ectopic adrenal cortical tissue may be found adjacent to, but not within, the testis [97]. Adrenal cortical hormone producing tumors arising within the testis probably represent adrenal cortex-like differentiation in Leydig cell tumor or steroid cell differentiation of tumors derived from pluripotential stromal cells [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) [166] and in the adrenogenital syndrome.

15.4.2 Carcinoid Tumors

Testicular carcinoid tumors are rare tumors and can represent a primary testicular neoplasm, a carcinoid tumor component within a testicular teratoma, or a metastatic carcinoid tumor involving the testis [167]. While primary carcinoid tumors are more common than metastases, patients with testicular carcinoid tumors *should be* evaluated for the presence of carcinoid tumor at other sites as well [167].

Primary carcinoid tumors of the testis comprise only 0.23% of testicular tumors and they typically are not associated with the carcinoid syndrome [168, 169]. They commonly present at an older age than most germ cell tumors, with a mean of 43 years in one series of 10 cases [168]. Single cases have been reported in a 10-year-old child [170] and a 19-year-old man [171]. A single case associated with an undescended testis [172] and a single-bilateral case [173] have been reported. Serum AFP, hCG, and serotonin are generally not elevated [168, 174]. Ultrasound demonstrates a solid mass with calcifications, but this finding is not specific [175, 176]. In one case, there was carcinoid syndrome with diarrhea and postprandial sweating at the time of presentation with a testicular mass [177]. A second patient had watery diarrhea that resolved following orchiectomy. This case was associated with an elevated serum serotonin level [178]. Facial flushing and sweating in the absence of diarrhea have also been reported [170, 179]. One tumor was reported in association with peptic ulcer disease, but the association may be coincidental [180].

Histologically, these tumors closely resemble midgut insular carcinoid tumors. The tumor cells are arranged in interconnecting nests, acini, and solid areas (Fig. 15.11). 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 [181].

The tumor cells are argentaffin and argyrophil positive and periodic acid-Schiff positivity may be noted in the acinar structures. Immunohistochemical staining for low molecular weight cytokeratin, neuron-specific enolase, chromogranin, and synaptophysin are positive [174, 182–184]. Serotonin, substance P, gastrin, and vasoactive intestinal peptide immunoreactivity may also be demonstrated [183, 185].

The primary clinical and pathologic considerations following a testicular carcinoid tumor diagnosis are: exclusion of the possibility of metastasis from another site and exclusion of an associated germ cell tumor component [186, 187]. 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 [188]. Octreotide scintigraphy may be used to exclude extratesticular carcinoid tumors [189]. More recently, computed tomography, urinary 5-hydroxyindoleacetic acid measurement, somatostatin receptor scintigraphy, and video capsule endoscopy have all been considered most useful to evaluate patients for carcinoid tumors at other sites [167]. Metastatic carcinoid originating from the ileum may present as a mass within [168] or adjacent to [190, 191] 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 [168]. 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 [181, 192]. Inguinal orchiectomy alone is *adequate* treatment [169]. In a recent review of 44 cases of primary testicular carcinoid tumor not associated with a teratoma, 7 (16%) developed metastasis [167]. One case report described widespread metastasis at the time of orchiectomy [193] while another reported metastatic spread with associated carcinoid syndrome 17 years following orchiectomy [194].

Although the majority of testicular carcinoid tumors are unassociated with teratoma, they may also arise as a component of mature teratoma [168, 195–197], and carcinoid tumors arising in testicular teratomas may share the isochromosome 12p cytogenetic abnormality with the teratoma [198]. Additionally, although most testicular carcinoids, including virtually all of the primary pure carcinoid tumors, have lacked associated intratubular germ cell neoplasia [126, 184], some of those associated with teratoma have had adjacent intratubular germ cell neoplasia [198]. Enteroendocrine cells have been identified in up to 21% of testicular teratomas [199, 200]. 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 [201].

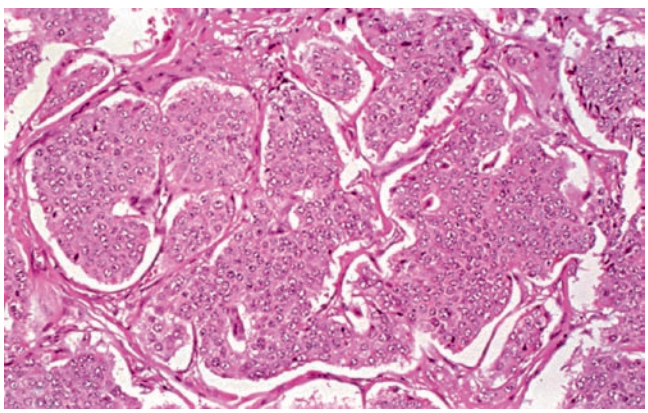

Fig. 15.11 Carcinoid tumors of the testis resemble midgut carcinoid tumors with interconnecting nests of tumor. The cells have neuroendocrine nuclear features and finely granular eosinophilic cytoplasm

15.4.3 Paraganglioma

Paraganglioma of the spermatic cord, but not within the testis, has been the subject of single case reports [202, 203]. Histologically, it appears as nests of polygonal cells in a "Zellballen" pattern. The cells have abundant eosinophilic gran-

ular cytoplasm. The tumor cells are immunohistochemically positive for neuron-specific enolase, chromogranin A, and synaptophysin. Sustentacular cells surrounding the nests are positive for S-100 protein. These tumors have not been associated with clinically significant hormonal manifestations.

15.4.4 Primitive Neuroectodermal Tumor

Primitive neuroectodermal tumor (PNET) has been described arising in testicular germ cell tumors (Fig. 15.12) [204, 205]. One report of 29 cases of PNET arising in a testicular germ cell neoplasm details the use of immunohistochemistry to further subclassify these tumors into neuroblastoma, medulloepithelioma, peripheral neuroepithelioma, and ependymoblastoma [206]. The PNET component is composed of small, round, blue cells and may be seen in the primary tumor, metastases, or both. 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. Although there remains the theoretical possibility that excess catecholamine production could develop from PNETs showing neuroblastic differentiation, we are *not* aware of reports of this phenomenon.

15.4.5 Neuroblastoma

In a series of 11 cases of neuroblastoma involving the testis, all proved to be metastatic on further evaluation [207]. One case of congenital paratesticular neuroblastoma likewise subsequently proved to be secondary from an adrenal primary [208]. A single case of primary testicular neuroectodermal tumor compatible with neuroblastoma has been reported [209]. 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.

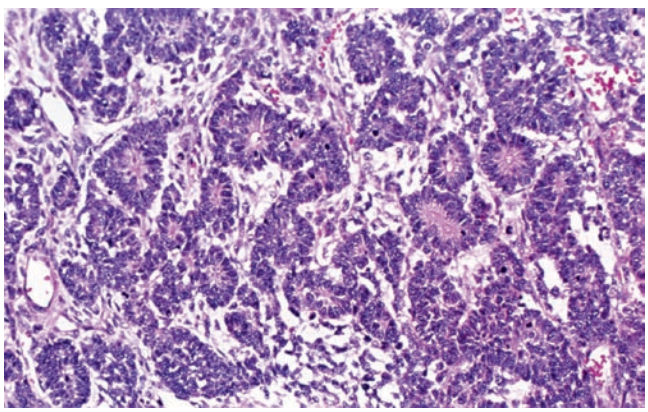

Fig. 15.12 Primitive neuroectodermal tumors are composed of cells with scant cytoplasm and crowded nuclei with fine chromatin. Rosette-like structures are common

15.4.6 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 [210, 211]. These have lacked paraneoplastic and endocrine manifestations, but these features are obviously possible given the potential of small cell carcinoma of the lung and other sites to produce such manifestations.

15.5 Genetic Syndromes

15.5.1 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 [135]. Several cases of Sertoli cell tumors in boys with Peutz–Jeghers syndrome have been reported [212–215]. These tumors may be bilateral and multicentric and are associated with gynecomastia, rapid growth, and advanced bone age [212, 216]. Although many have been classified as the large cell calcifying Sertoli cell tumor subtype of Sertoli cell tumor [135], in our opinion, most represent a distinctive form of intratubular neoplasia that we have descriptively termed "intratubular large cell hyalinizing Sertoli cell neoplasia" (Fig. 15.13). There also appear, however, to be some uncommon legitimate examples of the large cell calcifying Sertoli cell tumor in Peutz–Jeghers patients. Patients may have testicular enlargement in the absence of a discrete mass and, on biopsy, intratubular Sertoli cell proliferations may be seen [215, 217] These lesions have a low risk of progression on follow-up, but occasional cases may progress to invasive large cell Sertoli cell tumors [215].

15.5.2 Carney Complex

Carney complex is an autosomal dominant multiple neoplasia syndrome that includes primary pigmented nodular

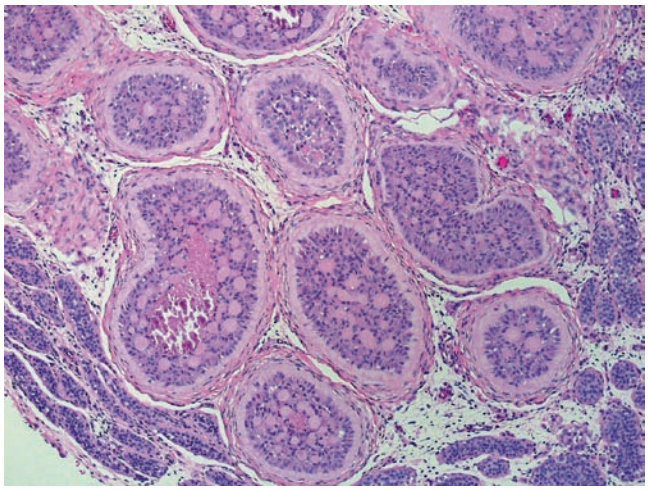

Fig. 15.13 Intratubular large cell hyalinizing Sertoli cell neoplasms in a patient with Peutz–Jeghers syndrome

adrenocortical disease, pituitary adenomas, thyroid adenomas or carcinomas, cardiac myxomas, gastric stromal tumors, ovarian cysts, and other tumors. The complex results from, in most cases, a 2p or 17q mutation [135]. Testicular manifestations are present in more than half of male Carney complex patients [135]. The most common testicular tumor, seen in one-third of male patients, is large cell calcifying Sertoli cell tumor [218]. This tumor may be accompanied by symptomatic hormone production with gynecomastia in prepubertal boys [218, 219]. Other endocrine manifestations may result from synchronous tumors at other sites, such as Cushing's syndrome due to an adrenal adenoma [219]. 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 [218].

15.5.3 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 [220]. Cryptorchidism, but not the increased gonadotropin levels associated with Klinefelter syndrome, is believed to be associated with testicular neoplasia [221]. The tumors seen in cryptorchidism, XY gonadal dysgenesis, and testicular feminization are generally germ cell tumors or gonadoblastomas and typically lack endocrine manifestations [221].

15.6 Other Conditions

15.6.1 Cryptorchidism

Testicular pathology occurs in a variety of other endocrine and developmental conditions but, with the exceptions of biopsies performed on cryptorchid testes or as a component of the evaluation of infertility, this pathology is rarely evaluated histologically. Cryptorchidism is a well-recognized risk factor for testicular cancer and infertility [222] and biopsy may be performed to evaluate the organ for the presence of IGCNU. It is possible that cryptorchidism, infertility, and testicular carcinoma may all be components of a single developmental disease, testicular dysgenesis syndrome [11, 13, 14]. The majority of cases of cryptorchidism have *no* apparent etiology [12]. Testosterone and insulin-like factor 3 are involved in regulation of testicular descent and mutations in the genes for insulin-like factor 3, for the insulin-like factor 3 receptor, and for the androgen receptor gene are responsible for a minority of cases of cryptorchidism [222]. Mutations of the genes for insulin-like factor 3 or its receptor can be identified in 4–5% of patients with cryptorchidism [223]. Environmental factors may also play a role [11, 222, 224], but other investigators have questioned this assumption [225]. Phthlates, synthetic compounds present in plastics which cause Leydig cell hypoplasia and suppress testosterone and insulin-like growth factor expression [10, 13], have received recent attention because of the testicular dysgenesis-like syndrome that they produce in rats [9] and because of epidemiologic evidence that they may be related to testicular dysgenesis syndrome in humans [10]. In mice, estrogenic hormones inhibit fetal Leydig cells and result in cryptorchidism through a pathway involving estrogen receptor alpha [226].

15.6.2 Other Disorders

Testicular microlithiasis, which may be diagnosed with testicular ultrasound evaluation, is considered, at least by some, as a potential risk factor for the subsequent development of a germ cell tumor, most likely because of its common association with testicular atrophy or "dysgenesis". Tubular atrophy, oligospermia, and Leydig cell hyperplasia are seen in myotonic dystrophy 1, relating to inactivation of the SIX5 allelle [99]. Prepubertal testis enlargement may be seen in McCune-Albright syndrome in association with increased serum inhibin B and anti-Mullerian hormone concentrations [227].

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Chapter 16
Endocrine Tumors of the Gastrointestinal System

Guido Rindi, Silvia Pizzi, Tiziana D'Adda, and Cesare Bordi

16.1 Introduction and Brief Historical Notes

The endocrine tumors of the gastrointestinal tract are made up of cells that are similar in phenotype to those belonging to the so-called diffuse endocrine system (DES) of the gut. The history of such enteroendocrine cells and of the 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 nineteenth and early twentieth centuries. Since their staining was attributed to the interaction with chromium salts [4], these cells were named as enterochromaffin cells [5]. The concurrent discovery of secretin by Bayliss and Starling in 1902 [6, 7] proved the gut as the source of blood-borne agents, "hormones," capable of eliciting physiological effects at a distance. In 1938, Feyrter described epithelial cells in different organs of the human body which 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 "diffuse endocrine system" (DES) [10]. More recently, in 1966, Pearse identified a group of cells containing amines and/or with the property of taking up amine precursors which 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 system (APUD) [12] which comprised, together with other types, the argentaffin,9 5-hydroxytryptamine-storing [13] cells of the gastrointestinal tract.

With the development of the concept of DES, a non-conventional, epithelial tumor with slow-growing attitude was identified in parallel and defined as "karzinoide" (carcinoid, i.e., carcinoma-like) 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].

16.2 The Endocrine Phenotype

Since the initial observation of Bayliss and Starling, a large number of hormones were identified in the gut such that the gastroenteropancreatic tract is now recognized as the largest endocrine organ of the whole human body [17]. The gut DES system is remarkably heterogeneous and is composed by 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 possible immune-barrier control. Such functional activities are exerted by synthesis and release of peptide hormones and biogenic amines specific to the individual cell types.

Gut cells of the DES share a number of antigens with neural cells, commonly defined as "neuroendocrine markers" [19], a finding that justifies the term "neuroendocrine" commonly used to connote DES cells and their tumors. For a general assessment of the neuroendocrine profile, the first methods developed and still in use include silver impregnation techniques, like Grimelius' stain (Fig. 16.1a) demonstrating argyrophilia and Masson-Fontana stain (Fig. 16.1b) demonstrating argentaffinity, i.e., 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, though effective and reproducible have now been largely substituted by immunohistochemistry for cytosol markers, like 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), like chromogranins and related fragments and protein 7B2 [27–30], or small synaptic-like vesicles (SSV) markers like

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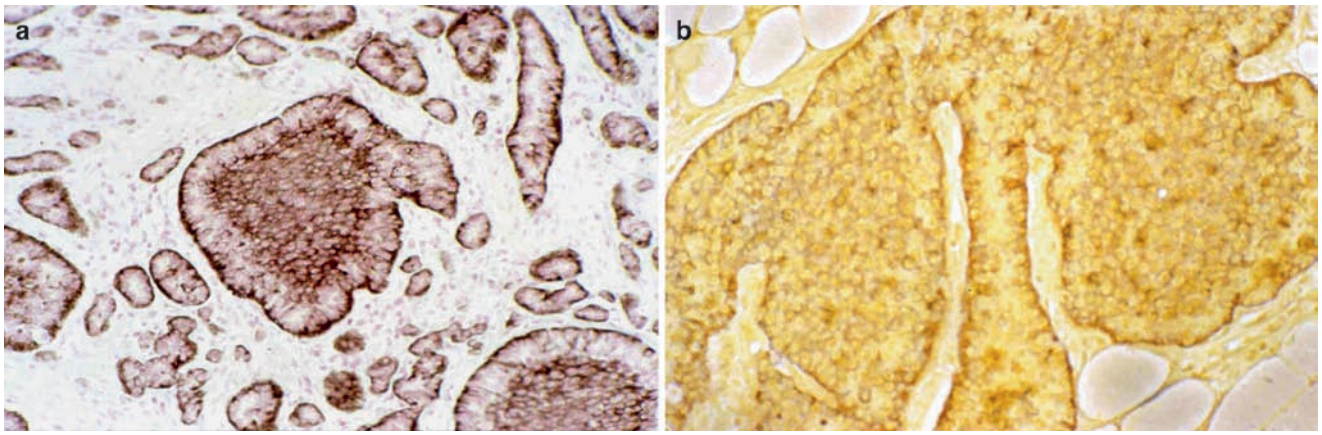

Fig. 16.1 Intense argentaffinity (**a**) and argyrophilia in EC cell tumors of the small intestine; note the islet structure with silver accumulation at the periphery of cells. Masson-Fontana (**a**) and Grimelius (**b**) silver impregnation methods

Table 16.1 Distribution of endocrine tumors in the gastrointestinal tract according to differentiation status and cell type [152]

				Intestine					
		Stomach		Small			Large		
Tumor type	Main cell	CF	An	D	J	I	Ap	C	R
Well-differentiated	D			+	+				
	EC	+	+	+	+	+	+	+	+
	ECL	+							
	G		+	+	+	+			
	L				+	+	+	+	+
Poorly differentiated	s/i	+	+	+	+	+		+	+

Modified from Rindi et al. 1999 [152].
CF: corpus-fundus; An: antrum; D: duodenum; J: jejunum; I: ileum; Ap: appendix; C: colon; R: rectum; +: presence of tumor; EC: enterochromaffin cell; ECL: enterochromaffin-like cell; PHH: persistent hyperinsulinemic hypoglycemia; s/i: small–intermediate cells.

synaptophysin [31–33]. Various other bioactive molecules were proven to be stored within LDCV granules. Two isoforms of the ATP-dependent vesicular monoamine transporter protein (VMAT1 and VMAT2) were identified [34]. Both isoforms are found in the adrenal medulla, but VMAT1 is only expressed in gut enterochromaffin (EC) cells and VMAT2 in the gastric enterochromaffin-like (ECL) cells, and in pancreatic islets and derived tumors [35–37]. More recently, peptide products of the neurotrophin–inducible gene *vgf* were identified in human DES cells with different distribution, likely reflecting cell-type-specific processing or different cell functional status [38].

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, defined as "specific markers" [18].

Gut endocrine cells and related tumors are also characterized by the production of several growth factors and by the expression of their relevant receptors [39]. This property may possibly explain some clinical manifestation of hyperfunctional syndromes associated with endocrine tumors (e.g., carcinoid heart disease) [40] and some histological aspects (e.g., smooth muscle cell proliferation, as often observed in gastric tumors) [39].

The somatostatin receptor subtype 2 (SSR2) is diffusely and abundantly expressed in gut endocrine cells and related tumors [41–45]. This feature is of great importance for both targeted diagnostic and therapeutic applications [46, 47]. To allow patient selection on pathology specimens, a practical diagnostic score was recently proposed based on a concordance study between in vivo assessment and SSR2A tissue immunohistochemical expression [48]. Of the five known dopamine receptors, (DR), D2R-like (DR2, DR3, and DR4) receptor expression was demonstrated in neuroendocrine cells and tumors [49, 50]. Given the documented efficacy of DR2 receptor-targeted therapy in endocrine tumors of pituitary and andrenal [51–53], the recent development of chimeric multireceptor ligands may open novel therapy options [54–56].

Considering the anatomical and functional heterogeneity of DES cells, it is not surprising that gut endocrine tumors constitute an heterogeneous group with remarkable differences regarding genetic background, functional properties, related clinical syndromes, clinico-pathological association, and prognosis (Table 16.1).

16.3 Histogenesis

Several evidences, both in experimental animals and in human conditions, link endocrine cells and endocrine tumors of gut and pancreas. Targeting endocrine cells in transgenic mice with constructs in which an endocrine gene promoter drives the expression of powerful oncoprotein-coding sequences resulted in endocrine tumors, both in pancreas and the intestine [57–59]. Similarly, the knock-out of the murine MEN1 gene in mice resulted in multiple endocrine tumors similar to the human condition [60, 61]. The recent discovery of a p27Kip1 gene-related heritable multiple endocrine tumor syndrome (MENX) allowed the identification of a similar condition in man, stressing the utility of genetic modeling studies [62]. In addition, in both experimental models and corresponding human conditions, a range of pre-neoplastic lesions of endocrine cells support a multi-step tumor development (for review, *see* [63]). The identification of key gene(s) involved in endocrine cell transformation is, however, still largely elusive.

16.4 Genetic Background

In general, the genetic background of endocrine tumors of the gastrointestinal tract has been poorly studied. The tumors investigated are relatively few, frequently lack tumor cell-type details and, finally, most investigations lack mutational analysis. These facts may, in part, reflect the paucity of proper, fresh frozen, material available for study. Some of the evidences available to date are briefly described below (for details, *see* also [64]).

A major step in understanding the genetic basis of gastrointestinal endocrine tumors was the identification of the gene, co-segregating with the multiple endocrine neoplasia type 1 (MEN1) syndrome, in which endocrine tumors of the pancreas, duodenum, and stomach are found [65]. Located on chromosome 11q13, the MEN1 gene is found to act as a tumor suppressor gene [66]. 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 chromosomal deletion (loss of heterozygosity, LOH) or point mutations. The MEN1 gene spans 9 kb and consists of 10 exons with a 1,830 bp coding region encoding a novel 610 amino acid protein, referred to as menin [67]. Menin localizes to the nucleus and inhibits the AP-1 transcription factor JunD [68]. Moreover, menin interacts with NF-κB proteins inhibiting NF-κB-mediated transcriptional activation [69].

Available data point to significant difference between tumors from foregut (stomach, duodenum, and upper jejunum), midgut, and hindgut derivatives. Such difference reflects the tumor involvement in *MEN1* 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 [70–74] and the upper intestine [71, 73, 75–78]. Gastric endocrine tumors with *MEN1* gene abnormality include poorly differentiated carcinomas (*see* following paragraph for tumor definitions) [74, 79]. Functioning G-cell tumors of the duodenum and relative metastasis composed the largest fraction of investigated intestinal tumors. Notably, the 11q deletions reported in the upper gut endocrine tumors (Fig. 16.2a) may display continuous losses up to the most distal marker investigated [74], as also seen in pancreatic endocrine tumors [80] (Table 16.2). Mutations of the *MEN1* gene were also reported in a fraction (about 30%) of sporadic tumors of the upper gut [77, 78, 81–83]. In addition, *MEN1* gene mutations were reported in one poorly differentiated endocrine carcinoma of the stomach [79]. The above evidence strongly suggests a significant role of *MEN1* gene, possibly as tumor-inducing defect in endocrine cells of the upper gut.

Conversely, relatively few tumor cases of midgut and lower gut have been investigated either for *MEN1* allelic loss or mutation resulting mostly negative for both analyses (Fig. 16.2b) [64]. This was confirmed by our study showing an overall 9% LOH rate of 11q markers investigated in 16 ileal, 6 appendicular, and 3 rectal well-differentiated endocrine tumors [80]. Notably, in this report 11q losses were of limited extension, if not interstitial, and consistently discontinuous (Table 16.2). *MEN1* gene mutation was found only in one of the 12 midgut endocrine tumors investigated so far [82, 83]. These evidences do not support a significant role of *MEN1* gene in midgut and lower gut endocrine tumors.

Other genes have been investigated though information is again rather 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 [84].

Methylation at 5′ regions of various genes has been demonstrated as a potential pathogenetic mechanism for endocrine tumor development in the gut. An analysis of multiple genes promoters revealed the frequent occurrence of *RASSF1A* promoter methylation on chromosome 3p [85]. This phenomenon was mostly restricted to foregut neoplasms including poorly differentiated carcinomas, and associated with malignancy [86, 87]. Promoter metilation 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 [88]. Similar findings were obtained in 4/9 midgut EC cell tumors [89].

No *p53* gene mutation was detected in well-differentiated gastric, small intestinal, and appendiceal “carcinoid,” while

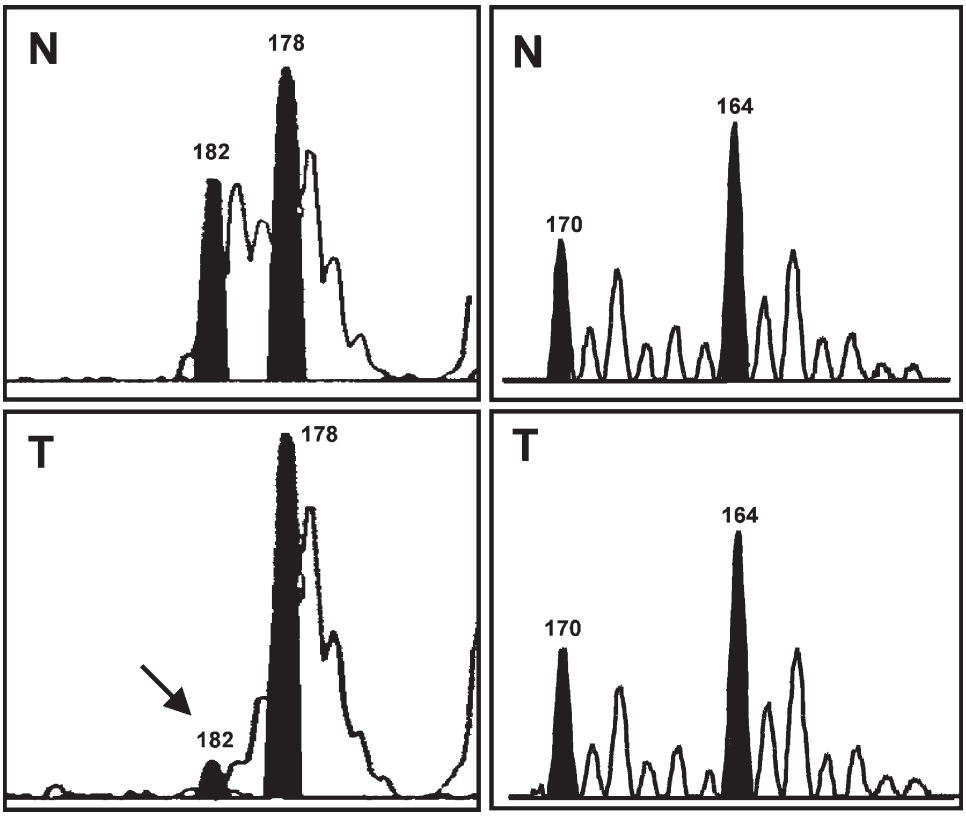

Fig. 16.2 *Left panels*. Allelic loss at the 11q13/MEN 1 locus in a type I gastric carcinoid found after PCR amplification of the microsatellite marker PYGM with almost complete disappearance of the peak corresponding to the larger allele (*arrow*) in the tumor tissue (T) as compared to normal tissue (N). *Right panels*. Absence of allelic loss at the same microsatellite marker in a midgut (ileal) carcinoid

Table 16.2 Comparison of llq allelic deletions in pancreatic (foregut, A) and mid–/hindgut (B) endocrine tumors, showing the specificity of high LOH frequency with consistent extension to the most telomeric marker in the pancreatic neoplasms [80]

A	Insulinomas						Others				MEN–1				Non–functioning												
Case no	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
BIM	*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 no.	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
BIM	*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 D'Adda et al. 2002 [80].

one out of nine tumors of the colon–rectum proved mutated [90]. Indeed *p53* gene abnormalities, like TP53 LOH at 17p and hyperexpression-accumulation, are mostly restricted to poorly differentiated endocrine carcinomas of the gut [86, 91–94].

The imbalance of chromosome 18, with 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 LOH and comparative genomic hybridization (CGH) studies [95–103]. 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 [92].

Extensive X-chromosome allelic deletions were found in all malignant gastric endocrine carcinomas investigated ($n=4$), while no significant loss was reported in benign tumors ($n=29$) [74]. In accordance with similar data obtained in pancreatic and lung endocrine tumors [104–106], such evidence supports an association between X-chromosome LOH and malignancy as limited to foregut endocrine tumors. Conversely, such association was not observed in malignant midgut endocrine tumors [104]. The minimal X chromosome region most frequently involved in LOH is located at Xq25–Xq26 [107].

CGH analysis also demonstrated the occurrence of chromosomal gains on several chromosomes including 4, 5, 7, 13, 14q, 16, 20, and 21, occurring with various frequencies [95–98, 102, 103]. The exact significance of such aberrations is, however, still elusive.

Pathway-oriented studies may prove to be either informative or useful. Therefore, the involvement of genes known to induce cell cycle arrest through the Rb-mediated checkpoint by inhibited cyclin D1 accumulation has been suggested in both well and poorly differentiated gut endocrine tumors [85–87]. The Wnt pathway also appears as an interesting candidate. Beta-catenin expression is indeed often abnormal in gut endocrine tumors, though mostly in the absence of consistent gene mutation of either beta-catenin or adenomatous polyposis coli (APC) [108–114]. Recently, a key role for APC was demonstrated via different alteration at different anatomical sites, namely promoter methylation and LOH, mostly restricted to gastroduodenal neoplasms and APC mutation in ileocolon [115]. By contrast, the Notch-1/hairy enhancer of split 1 (HES-1)/achaete-scute complex-like 1 (ASCL-1) signaling pathway is still a significant regulator of differentiation in gut endocrine tumor cells, suggesting a potential NOTCH-activating therapeutic strategy for gut endocrine tumors [116].

In conclusion, only for tumors of the upper gut consistent data indicate a pathogenetic role for the *MEN1* gene. Similar evidence is missing for ileum and large intestine tumors. Additionally, given that a substantial fraction of sporadic tumors at any gut location still lacks any *MEN1* gene involvement, other transformation events have to be hypothesized and explored. The present evidence suggests the involvement of yet unknown oncosuppressor gene(s) in 18q, especially in midgut tumors. *p53* gene involvement is consistently restricted to more aggressive carcinomas and mostly restricted to poorly differentiated endocrine carcinomas of the gut. Finally, the pattern of genetic abnormality appears to significantly differentiate tumors of stomach, pancreas, and duodenum as compared to that of ileum and colorectum.

16.5 Diagnosis

In agreement with the new formulation of the WHO classification [117], 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 further subdivided into two subgroups:

1. Well-differentiated endocrine tumors, which is further subdivided into 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.

The latter group comprises highly malignant tumors, virtually never associated with a hormone-related clinical syndrome and displaying an undifferentiated, though 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. Given the severe clinical implication of such diagnosis, discriminating between well-differentiated and poorly differentiated endocrine tumors is of paramount importance. Below we will 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.

A practical diagnostic algorithm for routine diagnostic pathology, independent of tumor site, includes (a) the identification of typical histology of such tumors, (b) the assessment of tumor cell differentiation status, (c) the identification of the prevalent endocrine cell type, when applicable and (d) the assessment of predictors of malignancy.

Histology. In general, well-differentiated tumors are characterized by bland features, organoid pattern, trabecular, glandular, acinar or mixed structures [118], tumor cell monomorphism with abundant variably eosinophilic cytoplasm, low cytological atypia, and low mitotic index (Fig. 16.3). On the contrary, poorly differentiated carcinomas usually display

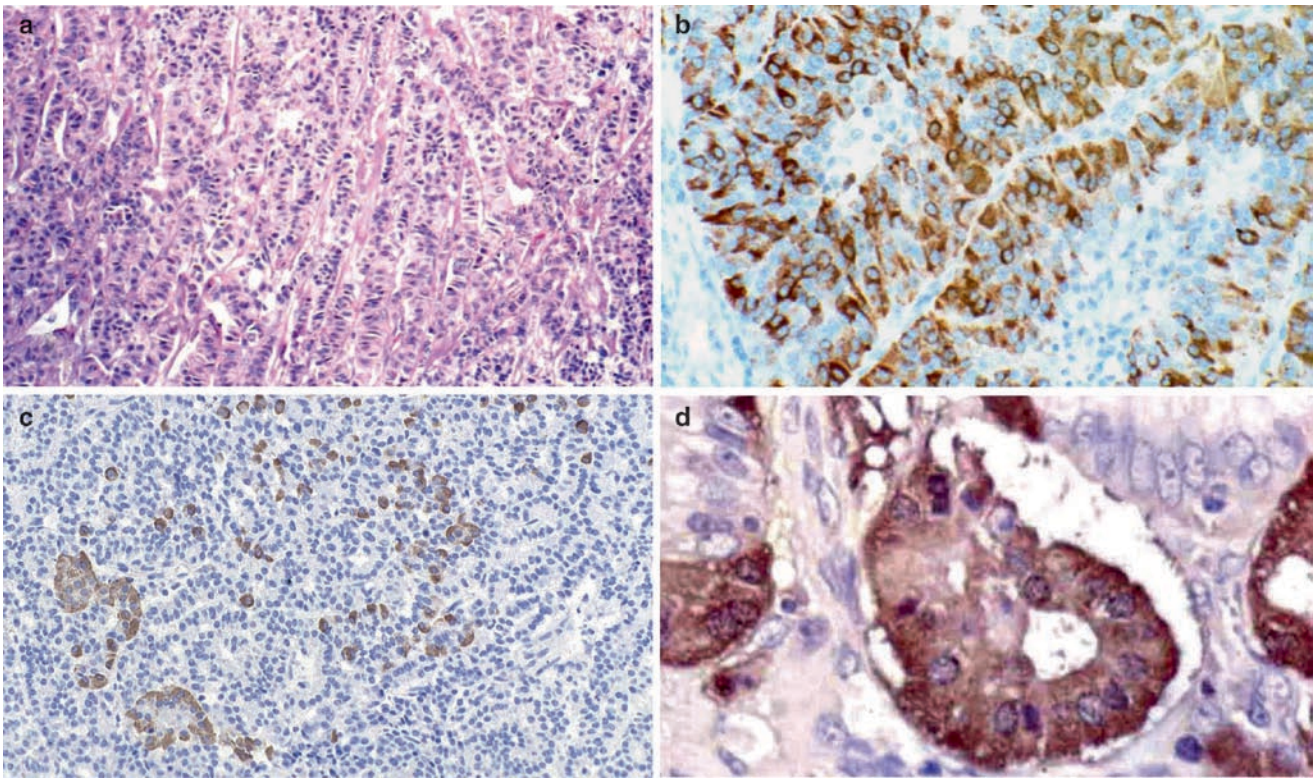

Fig. 16.3 Features of well-differentiated endocrine tumors of the gut. (**a**) Low power micrograph illustrating the mostly trabecular structure of a coecal tumor; note the regular and monomorph tumor morphology. (**b**) Diffuse though variably intense chromogranin A expression in a lymph-node metastasis 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 typical glandular structure. Haematoxylin and eosin (**a**); immunoperoxidase, ABC method; haematoxylin counterstain (**b–d**)

prevalent solid structure with abundant necrosis, often central, round tumor cell of small-medium size with severe cellular atypia and high mitotic index (Fig. 16.4) [117].

Differentiation status. Besides morphological criteria, it 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 protein gene product 9.5 (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* Sect. 16.2 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. 16.3b and c).

Conversely, poorly differentiated carcinoma cells are, in general, negative for chromogranin A, though diffusely positive for NSE, PGP 9.5, and synaptophysin (Fig. 16.4b and c). These features reflect the rarity of large dense-core, electron-dense granules observed in poorly differentiated carcinoma cells by electron microscopy [119]. The absence of chromogranin A and of hormone gene expression (*see* below) in poorly differentiated endocrine carcinoma cells may also be consistent with the demonstrated "On/Off" switch function exerted by chromogranin A gene in mammalian cells [120]. In the routine practice, diffuse and strong positive stain for at least two of the above-mentioned markers is required for the diagnosis of poorly differentiated endocrine carcinoma. High Ki67 index (Fig. 16.4d) and extensive p53 hyperexpression/accumulation (Fig. 16.4e) are also additional features, typical of the highly malignant poorly differentiated endocrine carcinoma (*see* following paragraph).

Tumor cell typing. In well-differentiated endocrine tumors, hormone-specific sera (specific markers) readily identify specific tumor cell types (Fig. 16.3d). This assessment allows the subclassification of the tumor cell-type, either in the presence or in the 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

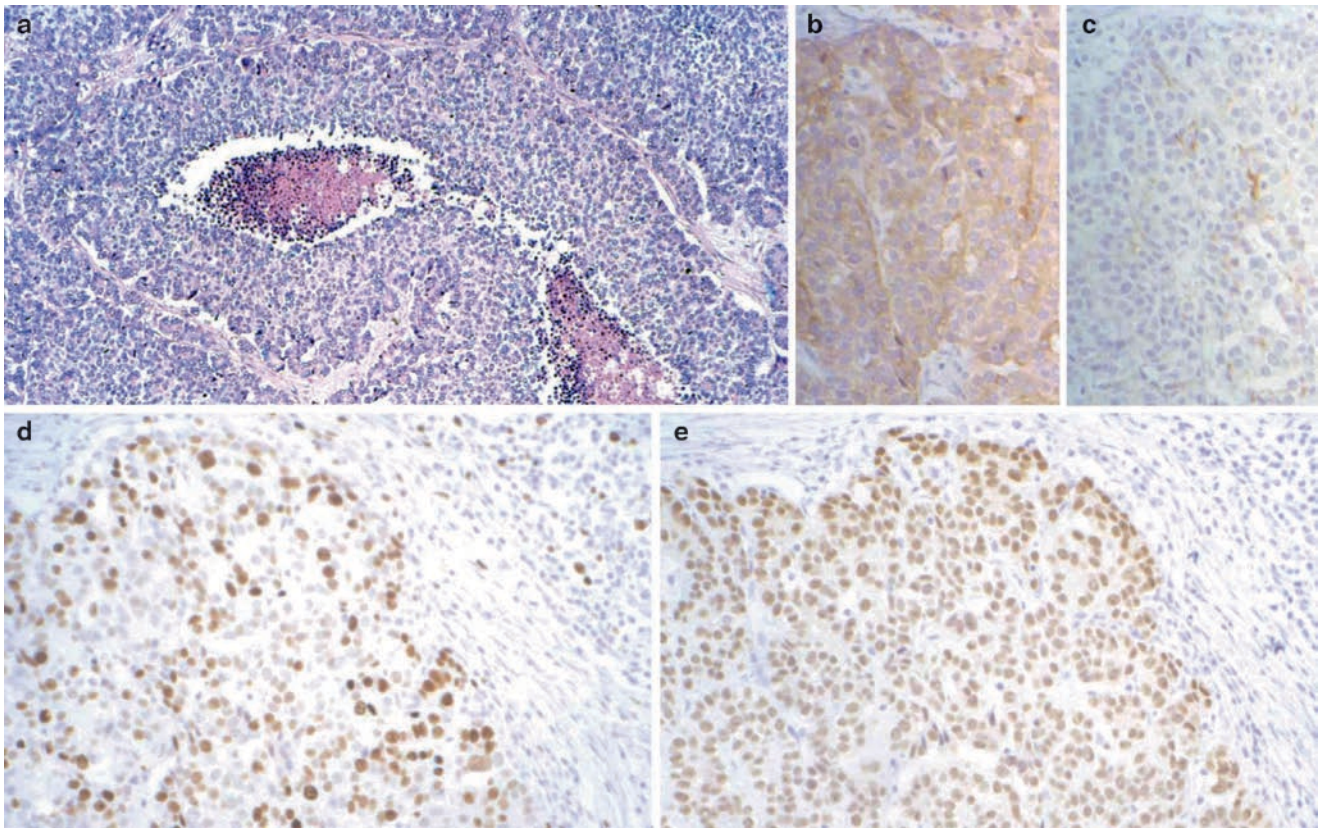

Fig. 16.4 Features of poorly differentiated endocrine tumors of the gut. (**a**) Solid structure with central necrosis of a poorly differentiated endocrine carcinoma of the stomach; the severely atypical tumor cells are tightly packed. (**b**, **c**) Intense and diffuse synaptophysin immunoreactivity (**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 Ki67 (**d**) and p53 (**e**); note that a significant fraction of tumor cells are Ki67 positive while almost all of them display accumulation/hyperexpression of p53; same case shown in (**b** and **c**). Haematoxylin and eosin (**a**); immunoperoxidase, ABC method; haematoxylin counterstain (**b**–**e**)

endocrine tumors with proven malignancy at diagnosis, i.e., presenting synchronous metastasis and/or deep wall invasion [117]. 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 evidences 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 (the reader is suggested to refer to the WHO blue book for reference [117]). Finally, the malignant potential is not related to tumor cell type.

A number of clinico-pathological parameters, some of which are included in the new WHO classification, 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 (at all sites except the appendix); angioinvasion and invasion of perineural spaces; solid, non-organoid structure; necrosis; overt cell atypia; ploidy status (aneuploidy correlates with poor prognosis) [121]; more than two mitosis in 10 microscopic fields at high power (HPF); Ki67 index of more than 2% or of more than 100 in 10 HPF (Fig. 16.4d); loss of chromogranin A immunoreactivity, argyrophilia or hormone expression, and nuclear *p53* protein hyperexpression/accumulation (Fig. 16.4e).

The predictive value of some of these variables was demonstrated in retrospective studies of large series of pancreatic and gastric tumors [93, 121–123]. In specific, tumor size, angioinvasion, mitotic index, and Ki67 emerged as an independent predictor of malignancy and survival in gastric endocrine tumors [93]. However, mid- and lower-gut endocrine tumors have not been systematically investigated for most of the above variables and, for some of them, the published data are scant and contradictory. In a small series of midgut well-differentiated tumors, Ki67 index was initially suggested as a potential marker of

Table 16.3 ENETS grading proposal for gut endocrine tumors

Grade	Mitotic count (I0HPF)	Ki-67 index (%)
G1	<2	≤2
G2	2–20	3–20
G3	>20	>20

malignancy [124, 125]. However, in a subsequent study we failed to confirm this indication showing Ki67 values similar to those of benign digestive endocrine tumors [126]. In rectal carcinoids, a single study showed a very low Ki67 index (median, 0.62%) [127]. Although unrelated to clinico-pathologic features, such as infiltration of the muscularis propria, lympho- or angioinvasion, or lymph node metastases, the Ki67 index was significantly higher in tumors larger than 0.5 cm in size and positively correlated with TGFα expression [127]. Finally, in small series of midgut carcinoids, the ploidy status appear not to correlate with malignancy [128, 129], as was confirmed by a recent investigation [104]. By contrast, aneuploid status was found in three malignant rectal carcinoids [130].

The major limit of the above-reported data is the small size of the tumor series investigated. Extensive work on large and homogeneous tumor series is, therefore, required to better characterize malignancy-associated parameters for intestinal endocrine tumors.

Recently, an attempt to simplify the current WHO classification was developed by the European Neuroendocrine Tumor Society. The need for diagnostic standard and patient stratification led to the development of two complementary diagnostic instruments, a grading system based on the assessment of the proliferation fraction (Table 16.3) and an organ-specific Tumor Node Metastasis (TNM) staging classification (Tables 16.4–16.8) [131, 132]. This novel proposal stems from the WHO classification incorporating all histopatological diagnostic critera, however, embedding the malignancy concept within the diagnosis of gastrointestinal endocrine tumor. This proposal needs confirmation on large clinical series. Data published so far in foregut neoplasms including those from stomach and duodenum support its use [133].

Table 16.4 ENETS TNM/staging classification proposal for endocrine tumors of the stomach

TNM				
T	Primary tumor			
TX	Primary tumor cannot be assessed			
T0	No evidence of primary tumor			
Tis	In situ tumor/dysplasia (<0.5 mm)			
Ti	Tumor invades lamina propria or submucosa and <1 cm			
T2	Tumor invades muscularis propria or subserosa or >1 cm			
T3	Tumor penetrates serosa			
T4	Tumor invades adjacent structures			
Any T add (m) for multiple tumors				
N	Regional lymph nodes			
NX	Regional lymph nodes cannot be assessed			
N0	No regional lymph node metastasis			
N1	Regional lymph node metastasis			
M	Distant metastasis			
MX	Distant metastasis cannot be assessed			
M0	No distant metastases			
M1*	Distant metastasis			
*Specific sites defined according to [153]				
Stage				
Stage	0	Tis	N0	M0
Stage	I	T1	N0	M0
Stage	IIa	T2,	N0	M0
	IIb	T3	N0	M0
Stage	IIIa	T4	N0	M0
	IIIb	Any T	N1	M0
Stage	IV	Any T	Any N	M1

16.6 Differential Diagnosis

Well-differentiated endocrine tumor. Independent of 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 (PAS/Alcian blue or PAS after diastase digestion) and negative immunohistochemistry for endocrine markers support the diagnosis of conventional adenocarcinoma. Conversely, the presence of rare carcinoma cells that are positive for endocrine markers (e.g., silver impregnation or chromogranin A) is a frequent finding [134, 135], irrelevant though for the diagnosis of adenocarcinoma as well as for the clinical outcome of the patient [136]. 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 occurs between poorly differentiated endocrine carcinoma and non-endocrine 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 because of 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 immunohistochemmistry for general markers of endocrine differentiation (namely, sinaptophysin, NSE or PGP9.5) in the majority of carcinoma cells support the diagnosis of poorly differentiated adenocarcinoma.

Table 16.5 ENETS TNM/staging classification proposal for endocrine tumors of the duodenum/ampulla/proximal jejunum

TNM	
T	Primary tumor
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Tumor invades lamina propria or submucosa and site <1 cm*
T2	Tumor invades muscularis propria or subserosa or >1 cm
T3	Tumor invades pancreas or retroperitoneum
T4	Tumor invades peritoneum or other organs
Any T add (m) for multiple tumors	
*Tumor limited to ampulla of Vater for ampullary gangliocytic paraganglioma	
N	Regional lymph nodes
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis
M	Distant metastasis
MX	Distant metastasis cannot be assessed
M0	No distant metastases
M1*	Distant metastasis
* Specific sites defined according to [153]	

Stage				
Disease stages				
Stage	I	T1	N0	M0
Stage	IIa	T2	N0	M0
	IIb	T3	N0	M0
Stage	IIIa	T4	N0	M0
	IIIb	AnyT	N1	M0
Stage	IV	Any T	Any N	M1

Table 16.6 ENETS TNM/staging classification proposal for endocrine tumors of lower jejunum and ileum

TNM	
T	Primary tumor
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Tumor invades mucosa or submucosa and size 0.1 cm
T2	Tumor invades muscularis propria or subserosa or size >1 cm
T3	Tumor invades subserosa
T4	Tumor invades peritoneum/other organs. For any T add (m) for multiple tumors
N	Regional lymph nodes
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis
M	Distant metastasis
MX	Distant metastasis cannot be assessed
M0	No distant metastases
Ml	Distant metastasis
* Specific sites defined according to [153]	

Stage					
Disease stages					
Stage	I	T1	N0	M0	
Stage	IIA	T2	N0	M0	
	IIB	T3	N0	M0	
Stage	IIIA	T4	N0	M0	
	IIIB	Any	T	N1	M0
Stage	IV	AnyT	AnyN	M1	

Table 16.7 ENETS TNM/staging classification proposal for endocrine tumors of the appendix

TNM	
T	Primary tumor
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Tumor 0.1 cm invading submucosa and muscularis propria
T2	Tumor 0.2 cm invading submucosa, muscularis propria and/or minimally (up to 3 mm) invading
T3	Tumor >2 cm and/or extensive (>3 mm) invasion of subserosa/mesoappendix T4 Tumor invades peritoneum/other organs
N	Regional lymph nodes
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis
M	Distant metastasis
MX	Distant metastasis cannot be assessed
M0	No distant metastases
M1*	Distant metastasis
* Specific sites defined according to [153]	

Stage				
Disease stages				
Stage	I	T1	N0	M0
Stage	IIA	T2	N0	M0
	IIB	T3	N0	M0
Stage	IIIA	T4	N0	M0
	IIIB	AnyT	N1	M0
Stage	IV	Any T	Any N	M1

Table 16.8 ENETS TNM/staging classification proposal for endocrine tumors of colon/rectum

TNM	
T	Primary tumor
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Tumor invades mucosa or submucosa
	Tia size <1 cm
	Tib size 1–2 cm
T2	Tumor invades muscularis propria or size >2 cm
T3	Tumor invades subserosa/pericolic/perirectal fat
T4	Tumor directly invades other organs/structures and/or perforates visceral peritoneum
For any T add (m) for multiple tumors	
N	Regional lymph nodes
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis
M	Distant metastasis
MX	Distant metastasis cannot be assessed
M0	No distant metastases
M1	Distant metastasis
* Specific sites defined according to [153]	

Stage				
Disease stages				
Stage	IA	T1a	N0	M0
Stage	IB	T1b	N0	M0
Stage	IIA	T2	N0	M0
	IIB	T3	N0	M0
Stage	IIIA	T4	N0	M0
	IIIB	AnyT	N1	M0
Stage	IV	Any T	Any N	M1

16.7 Well-Differentiated Tumor Types

16.7.1 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, they are composed of histamine-producing ECL cells of the oxyntic mucosa and produce histamine and molecules involved in its synthesis and intracellular processing, such as histidine decarboxylase and vesicular monoamine transporter type 2 (VMAT2). In addition, rare tumor cells may express other hormones, such as serotonin, gastrin, and ghrelin [119, 137, 138].

Three independent types of ECL cell tumors have been identified based on the associated pathological conditions [119].

(a) 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 [139]. Type I tumors more frequently affect the female gender (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 lymphnodes are rare (less than 5%) and to distant sites exceptional. No cases of tumor-related death are on record [140]. From the clinical point of view, type I ECL cell carcinoids are consistently non-functioning.

(b) Type II tumors are associated with multiple endocrine neoplasia type 1 (MEN-1), usually with Zollinger-Ellison syndrome (ZES), and account for about 6% of ECL cell carcinoids. Usually multiple, Type II tumors are larger than those of Type I, though overall below 1.5 cm in size in 73% of cases [140]. 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 [140]. The prognosis of these tumors is usually good but cases with very aggressive course have been described [72]. In general, however the patient's prognosis depends more on the background MEN1 setting. From the clinical point of view, Type II ECL cell tumors are non-functioning, the associated ZES being caused by the concomitant gastrinomas of the pancreas or, more often, of the duodenum.

(c) 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 predominance for the male gender (74%) [140]. Type III tumors are usually single and in 33% of cases larger than 2 cm in diameter. Infiltration of the muscularis propria is observed in 76% of the cases and of the serosa in 53%. Though most type III tumors are histologically typical carcinoids, more atypical features may be found especially in neoplasms exceeding 2 cm in size [93, 141]. Metastases occur in three-fourth of patients, especially in tumors with atypical features, and are often located in the liver. Tumor-related death is observed in 27% of cases with a median survival of 28 months [140]. 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 cyanotic 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 [142].

More recently, multiple gastric endocrine neoplasms developing in two hypergastrinemic patients void of chronic atrophic gastritis or MEN1/ZES were described [143, 144]. The two cases reported were proposed as examples of a novel gastrin-dependent tumor type, with defective parietal cell function, such as pathogenetic mechanism and a yet-unknown genetic basis.

16.7.2 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-third of all duodenal neuroendocrine tumors, duodenal G-cell tumors are preferentially located in the proximal duodenum and are usually small (<1 cm in diameter) [145]. Metastases are common, even in 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 multi-centric and may escape detection even at surgery [146]. Both sporadic and MEN-1 associated duodenal G-cell tumors are often but not always associated with the 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 [147]. They are malignant (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.

16.8 Small Intestine–Ileum and Caecum

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 caecum, EC cell tumors are characterized by an expression of serotonin and substance P. Usually larger than 1 cm in size at diagnosis, EC cell tumors are multiple in up to 40% of cases and tend to deeply infiltrate the muscular wall with frequent metastases to lymph node [145]. 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 [148]. Due 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 [149].

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 as their midgut counterpart. In contrast, appendix EC cell tumors appear to originate from the submucosal neuroendocrine complexes closely associated with Schwann-like, S-100 immunoreactive, sustentacular cells [145, 148]. 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 larger than 2 cm in size or infiltrating the mesoappendix are potential source of metastasis.

16.8.1 Large Intestine

Similar to what has been 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* above) are reported.

Rectal L cell tumors. These tumors are usually composed of L cells producing peptides of the glucagon-glicentin and PP-PYY families [150, 151]. In this respect, rectal carcinoids are completely 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. In the common diagnostic practice, it is not infrequent to observe negative immunoreactivity using current commercial anti chromogranin A antibodies. This feature may tentatively refer to a peculiar pro-chromogranin A processing and/or abnormal antigen presentation. To overcome this aspect it is advised to perform the silver impregnation test, usually resulting strongly positive. From the clinico-functional standpoint, rectal carcinoids are usually silent. Their clinical behavior is mostly benign but tumors with more than 2 cm in size or infiltrating the *muscularis propria* may metastatize and pursue an unfavorable course.

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Chapter 17
The Endocrine Pancreas

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17.1 The Normal Endocrine Pancreas

17.1.1 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 as α and β. Just a few years later, other investigators described δ and the fourth type (pancreatic polypeptide (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.

17.1.2 Molecular Aspects of Endocrine Pancreas Development

In the human fetus, the primitive pancreas develops during the fifth week of gestation from two separate and independent endodermal primordial buds, one dorsal and one ventral, that eventually fuse to form the organ [14]. The dorsal pancreatic bud develops in close proximity to the notochord and to the dorsal aorta, whereas the ventral bud expands in close connection with the liver and bile duct epithelium that forms on the ventral face of the gut. Both buds contain precursors for all pancreatic cell types but give rise to different parts of the human pancreas. Precursor of the ventral bud gives rise to the posterior part of the head, or the uncinate process, whereas the dorsal bud contains the precursor for the anterior part of the head, the body, and the tail [15]. 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. α-, β-, and δ-cells appear at about the same period while PP-cells follow a few days later [14]. In addition, studies in mice have demonstrated an expression of peptide tyrosine–tyrosine (PYY) [16] and a transient expression of other gut hormones, such as secretin and gastrin, in early precursors of endocrine cells [17, 18], 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 weeks [19] 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 [20, 21].

17.1.2.1 Transcription Factors in Endocrine Pancreas Differentiation

Most of our knowledge on pancreas development originates from recent experiments performed in rodents and chickens. The molecular events supporting the early morphological changes that give rise to the formation of the dorsal and ventral pancreatic buds result from coordinated responses to extrinsic signals from the mesoderm. Recent studies have revealed that the dorsal and ventral programs of the pancreas are not completely identical and slightly asynchronous, due

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Table 17.1 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	No endocrine 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

to the two independent endodermal domains receiving distinct signals from their surrounding tissues [22]. Many transcription factors (Table 17.1) have been identified as crucial for normal pancreas development [23, 24]. They may be classified into two main sets. The first group of transcription factors characterizes the pancreatic anlage and absence of these factors affects both the endocrine and the exocrine lineage. The second group of transcription factors is involved in the differentiation of pancreatic cell types from protodifferentiated precursors. Mutations in genes of this second group perturb the development of either the endocrine or the exocrine compartment.

So far three transcription factors are known to belong to the first group: Pdx1, Hlxb9, and Isl1, the master regulator of pancreatic development and β-cell differentiation. Genetic lineage labeling in mouse has shown that, early in organ formation, Pdx1 expression marks a pluripotent population of cells that gives rise to all cell types of the neonatal pancreas (endocrine and exocrine) and the epithelium of the duodenum and posterior stomach [25, 26]. By contrast, pancreatic agenesis occurs in Pdx1 null mutant mice [27]. A characteristic feature of Pdx1 is the highly conserved homeodomain, a 61 amino acid domain that builds a helix-turn-helix motif with three α-helical segments, the third helix being the DNA-recognition helix. This transcription factor has been proposed to regulate the genetic expression of insulin and the glucose transporter GLUT-2 and to be essential for maintaining insulin storage [23]. Recent evidences suggest that the number of $Pdx1^+$ progenitors determines the final size of the pancreas and that its growth is fixed by the amount of $Pdx1^+$ progenitor cells [28]. The Notch signaling influences the final size of the pancreas, controlling the maintenance of $Pdx1^+$ progenitors in an uncommitted state and ensuring their expansion up to E12.5 in mouse [29]. The proper commitment of $Pdx1^+$ progenitors to a pancreatic fate depends on repression of endodermal sonic hedgehog (SHH), a potent repressor of islet formation and acinar brancing. This intercellular signaling protein is strikingly absent from the pancreatic endoderm, in contrast to high level expression in the adjacent gastric and duodenal endoderm. Hebrok et al. [30] have demonstrated that FGF2 and activin-B, members of the transforming growth factor β family, are secreted from the adjacent notochord and repress endodermal SHH expression in the dorsal region of the foregut destined to form the pancreas. Similarly, ventral foregut endoderm, which will give rise to liver and ventral pancreas is composed of a population of bipotential precursors able to adopt gene expression patterns characteristic of each organ [31]. In this model of liver development, FGF signaling from cardiac mesoderm is necessary for liver differentiation; abrogation of FGF signals allows the emergence of the endoderm's default pancreatic fate.

Moreover, two homeobox transcription factors, Hlxb9 and Isl1, are required for the initial induction of Pdx1 in pancreatic primordia. The homeobox transcription factor Hlxb9 is encoded by the Hlxb9 gene and its expression is observed in two distinct developmental phases: first during the evagination of the pancreatic buds and later in differentiation of β-cells. In mice homologous of null mutation of Hlxb9, the dorsal lobe of the pancreas fails to develop and lacks Pdx1 expression. Even though the ventral bud develops, it has small islets of Langerhans with reduced numbers of β-cells [32, 33]. The second factor determinant for Pdx1 induction is the Lim homeobox transcription factor Isl1. It was initially identified as a transcription factor binding the insulin gene enhancer region. During pancreas development Isl1 plays a role both as an extrinsic factor produced by mesodermal derivatives, and intrinsically expressed in pancreatic progenitors. The inactivation of Isl1 leads to altered Pdx1 expression and to an abnormal endocrine pancreas differentiation [34].

The differentiation into the mature endocrine or exocrine cell types relies on the activity of early factors involved in the segregation of specific lineages. Many studies have been conducted to determine the interactions between the pancreatic epithelium and the mesenchyma to terminally differentiate the hormone-secreting cells [22, 34]. Neurogenin 3 (Ngn3) is a bHLH protein which has a role as a key regulator of endocrine development. The Ngn3 protein is expressed exclusively in scattered cells in the pancreatic epithelium and it is clear

that Ngn3 positive cells play a major role in the embryonic development of the pancreas, giving rise to the whole pancreatic endocrine lineages [25]. Ngn3 is not coexpressed with any of the pancreatic hormones suggesting that it induces the differentiation of pancreatic precursors into the endocrine cell types, but does not directly activate the pancreatic hormone gene expression. Animals lacking Ngn3 fail to develop any endocrine cells, while exocrine tissue and pancreatic ducts are nearly normal [35]. Recent evidence has indicated that their population dramatically declines at birth and becomes undetectable in adult tissues. Mechanisms involved in endocrine specification down-stream of Ngn3 are still to be defined. During a later stage of endocrine pancreatic development, the specification into the mature endocrine cell types relies on the activity of early factors involved in the segregation of endocrine lineages (IA1, Arx, Pax4, Nkx2.2, MafB) and of late factors involved in the maturation of committed endocrine cells (Pax6, Isl1, Pdx1, Brn4, Hlxb9, mafA) [23].

17.1.2.2 Growth Factors in Endocrine Pancreas Differentiation

Growth factors are diffusible molecules that play a role in endocrine pancreas differentiation [36, 37]. 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 17.2) suggests that they may also play a biological role in regulating the cell functions of the islets.

Table 17.2 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	[50]
HGF	α-, β-cells	[447]
Activin A	α-cells	[61]
Activin B	δ-cells	[62]
TGFα	All types	[444]
$TGF\beta_{1-3}$	β-cells	[70]
VEGF	β-, PP-cells	[78, 79]
Growth factor receptors		
FGFR1	α-cells	[51]
FGFR2	β-cells	[49–51]
FGFR3	Rare	[51]
FGFR4	α-, β-, PP-cells	[51]
Met	β-cells	[447]
VEGFR1	Rare	[79]
Follistatin	β-cells	[60]

Recent studies [38–46] 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 [47]. Mice transgenic for the hybrid apolipoprotein E (Apo E)/FGF7 gene develop an increased number of peri-ductal islets and show a marked increase in insulin expression in ductule epithelial cells [48]. In addition, when the signals mediated by FGFR2 (the receptor specific for FGF7) are blocked in mice rendered transgenic for the dominant negative soluble receptor, no islets are found in the pancreas [49], suggesting that signals transduced via FGFR2 are important for pancreatic development. In this context, it is interesting to note that both FGF7 and FGFR2 [50, 51] have been detected in the islets of human adults.

Members of the transforming growth factor-β (TGFβ) superfamily also seem to be involved in the endocrine pancreas development [52]. The TGFβ superfamily includes several soluble factors, such as activins, inhibins and various TGFβ isoforms [53]. Activins induce the expression of pancreatic genes during early development [54] and in cultures from chick embryos [30]. In addition, activins, in combination with either betacellulin or HGF, are able to convert AR42J cells, representing a sort of pluripotent precursor cells, into insulin-secreting cells [55, 56]. 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 [57]. Various types of activins and inhibins have also been demonstrated in adult rat and human islet cells [58–62], 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 [63]. 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 [64, 65]. The actions of activins are in part modulated by follistatin, a protein not belonging to the TGFβ superfamily [53]. Follistatin itself is known to regulate the relative proportion of endocrine versus 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 [66]. Interestingly, follistatin has been localized in human and rat adult pancreatic islet cells [60, 67].

Different experimental approaches have been used to establish whether TGFβ isoforms could be implicated in pancreatic development. $TGF\beta_{1-3}$ isoforms are expressed during pancreas development [68, 69] as well as in the adult pancreas [70]. In E12.5 mouse embryo exogenous $TGF\beta_1$ induces a developmental block of acinar cells and, on the contrary, the stimulation of endocrine cell development, suggesting a role of $TGF\beta_1$ in the regulation of the

balance between the acinar and endocrine component of the pancreas [69].

The role of transforming growth factor-α (TGFα) in pancreatic endocrine cell differentiation is less clear. Studies on transgenic mice and rats have suggested that TGFα, either alone or in combination with gastrin, may be involved in endocrine pancreatic cell proliferation and differentiation [71, 72]. In general, members of the epidermal growth factor (EGF) family, which also includes TGFα, 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 [73].

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 [74].

VEGF has no effect on endocrine differentiation of primary cultures of rat duct cells [75] but its flk-1 receptor is needed for β-cell maturation from pancreatic duct cells [76]. In addition, VEGF and its two specific receptors (flt-1 and flk-1) have been identified in islet cells of normal mice [77] and humans [78, 79], where they may play a biological role regulating islet cell differentiation and intra-islet capillarization.

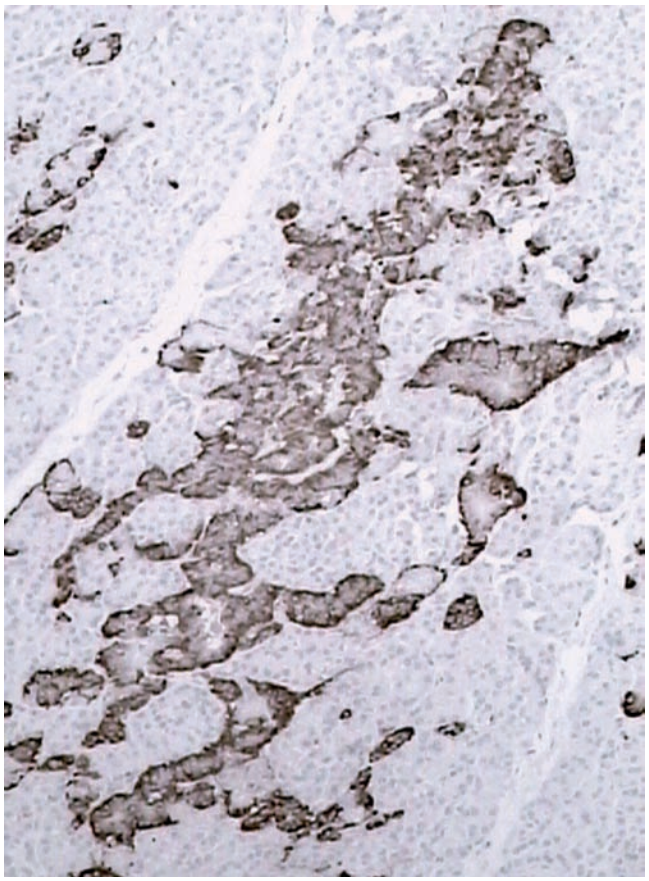

Fig. 17.1 PP-immunoreactivity in PP-rich islets of the ventrally-derived portion of the pancreatic head

17.1.3 Main Morphological Features of Adult Normal Islets

Although a small proportion (less than 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 mainly formed by thin trabeculae of perpendicularly oriented cells, most of which are PP-secreting (irregular or PP-rich islets) (Fig. 17.1) [80]. The four main types of islet cells show a different location within the islets (Fig. 17.2), different ultrastructural features (Fig. 17.3) and different hormone secretion. The main features of the four islet cell types are summarized in Table 17.3.

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 mainly located at the periphery of the islets [81]. 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 [82–85]. 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 (Fig. 17.3). The electron dense core primarily contains glucagon while other pro-glucagon derived peptides (i.e., glucagon-like peptides and glicentin) and chromogranins are stored in the pale halo [86, 87].

Insulin-producing β-cells represent the prevalent islet cell type of the regular islets (Fig. 17.2) and 20–30% of the cells forming the irregular islets [88]. They are easily identified by their immunohistochemical reactivity for insulin, proinsulin, C-peptide [9, 89, 90], and amylin (also called islet amyloid polypeptide-IAPP) [91]. Ultrastructurally, β-cells contain secretory granules either with a typical crystalloid core or a noncrystalline, finely granular compact core (Fig. 17.3). Crystalline granules contain mainly insulin while compact granules are considered immature and contain proinsulin [92].

δ-cells are identified by their immunoreactivity for somatostatin [93]. 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

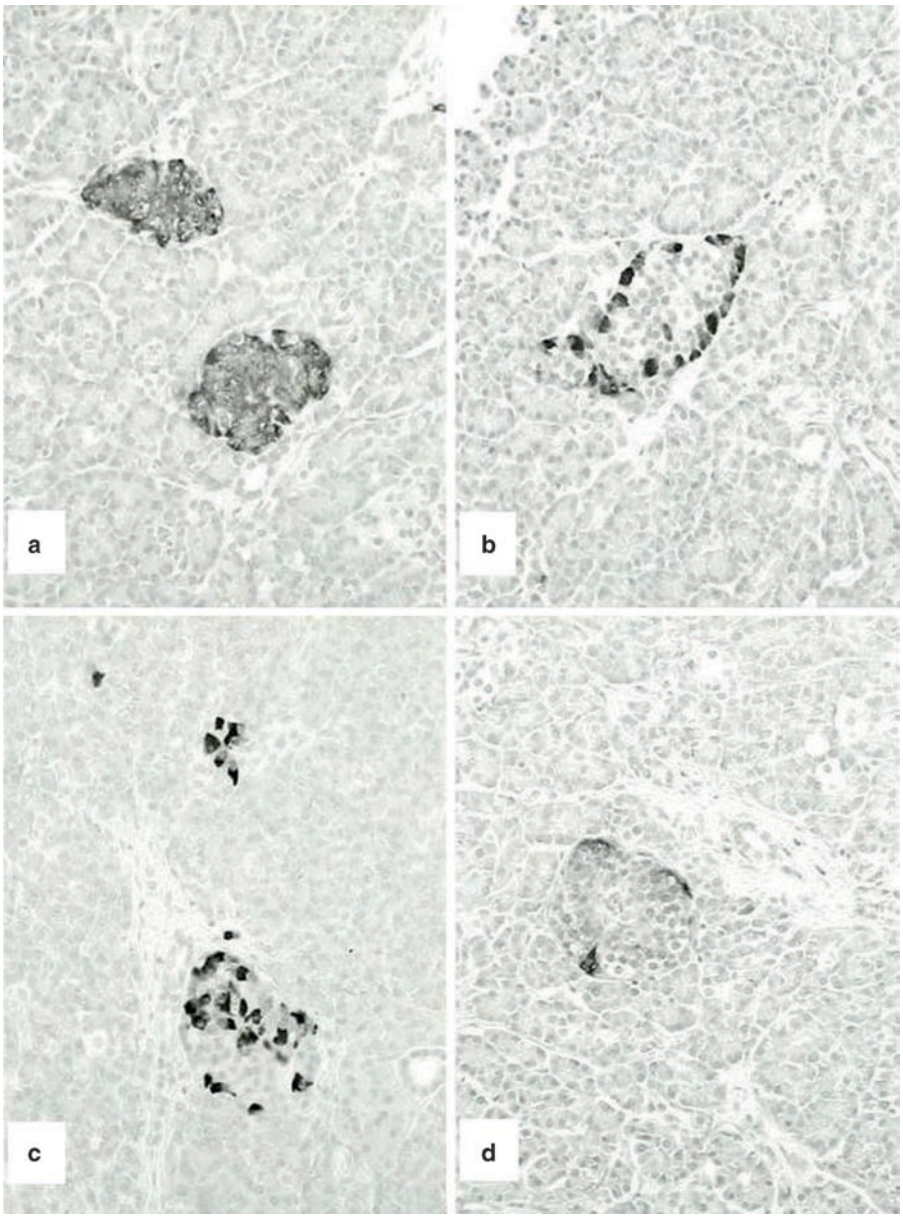

Fig. 17.2 Normal adult human pancreas. Immunoreactivities for insulin (**a**), glucagon (**b**), somatostatin (**c**) and PP (**d**) show the different distribution of β-, α-, δ-, and PP-cells in regular islets of the pancreas

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 (Fig. 17.3) [94]. δ-cells possess both short processes that contact endocrine cells and long processes that extend to intrainsular capillaries [95]. 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 [95].

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 (about 70%) cell type of irregular islets of the posterior part of the head (Fig. 17.1), but they account for only 2–5% of endocrine cells in the rest of the pancreas [80], 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 show small secretory granules [13].

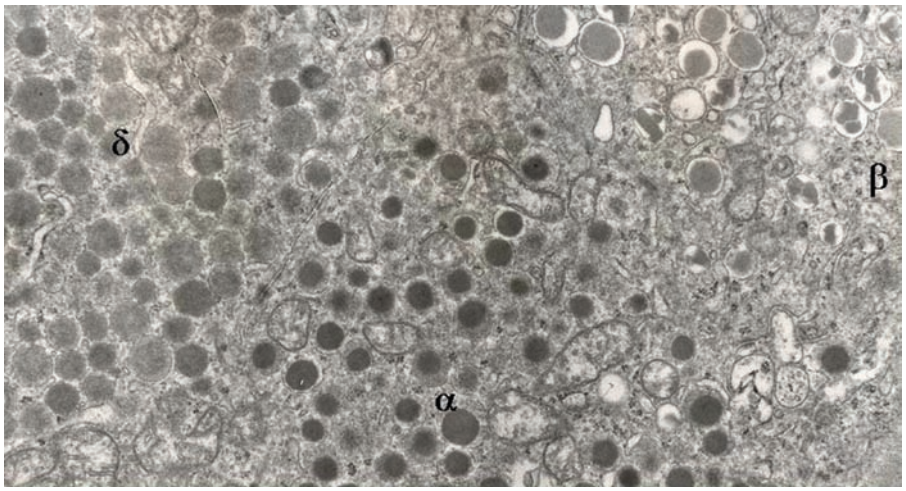

Fig. 17.3 Ultrastructural features of α-, β-, and δ-cells. α-cells (middle) have secretory granules with centrally or eccentrically located round electron-dense core, which is surrounded by a pale granular halo. β-cells (right) have secretory granules with both crystalline and noncrystalline core. δ-cells (left) show large secretory granules with uniform electron-dense core encircled by a tightly fitting membrane

Table 17.3 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 para-crystalline; 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

In addition to the four traditional known islet cell types, ghrelin-producing cells have been recently identified in rat pancreatic islets [96]. These cells seem to originate from duct cells and share lineage with glucagon cells. Ghrelin protein and mRNA have been also identified in β-cells of human pancreatic islets and in pancreatic endocrine tumors [97]; however in a different study [98] ghrelin immunoreactive cells were fairly represented in human fetal but not in adult pancreas and the ghrelin-reactive cells displayed ultrastructural features consistent with P/D1 cells of previous studies [99].

In addition to general endocrine markers and specific hormone peptides, islet cells have been found to express several growth factors (Table 17.2) and myosin XVA, an unconventional myosin protein, which seems to have a role in secretory granule movement [100, 101]. Moreover, islet cells express somatostatin receptors. Type 2 of somatostatin receptor was found to be expressed in all islet cell types, co-localizing with 70% of α-cells, 75% of β-cells and 55% of δ-cells, as well as type 3 and 5 [102, 103].

17.2 Diabetes Mellitus

17.2.1 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 Organization [104, 105] are reported in Table 17.4. Type 1 diabetes is due to β-cell destruction which, in more than 95% of cases, is caused by an autoimmune process [106]. Type 2 diabetes,

Table 17.4 Etiological classification of diabetes mellitus (modified from reference [105]).

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. Fridreich's ataxia
6. Huntington's chorea
7. Lawrence-Moon–Biedl syndrome
8. Monotonic dystrophy
9. Porphyria
10. Prader–Willi syndrome
11. Others
IV. Gestational diabetes mellitus

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 [107–109]. The pathogenesis and pathophysiology of diabetes with its clinicopathologic implications is a complex subject which is beyond the purpose of this chapter. Thus, the reader is referred to specific texts to get more information about this topic [110–112]. In this chapter we will restrict the discussion to the main morphological changes of pancreatic islets in type 1 and type 2 diabetes.

17.2.2 Type 1 Diabetes Mellitus

Type 1 diabetes mellitus accounts for about 10% of all cases of diabetes and occurs most often in young people, often manifesting itself in the form of ketoacidosis that can only be treated with insulin [111]. There are both geographic and race-specific differences in the incidence of type 1 diabetes [107, 109].

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 year thereafter, the weight and the size of the pancreas are normal [113–115]. However, after 2–5 years of diabetes, the pancreas gets smaller and, in some cases, the weight may be less than 50 g [113, 116]. This reduction in pancreatic weight is due to the atrophy of the exocrine parenchyma, which constitutes about 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 which may exert a trophic effect on acinar cells [117, 118]. 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 of onset has not been found [116].

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 Langerhans. Three types of islets can be identified [119, 120]: (1) 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) oval or round islets which are sharply demarcated from the exocrine parenchyma and tend to be larger than normal. They contain a normal number of β-cells which are large, degranulated and with nuclear hypertrophy, suggesting a functional hyperactivity; (3) 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 [121–123]. T-lymphocytes penetrate the islets from the periphery and

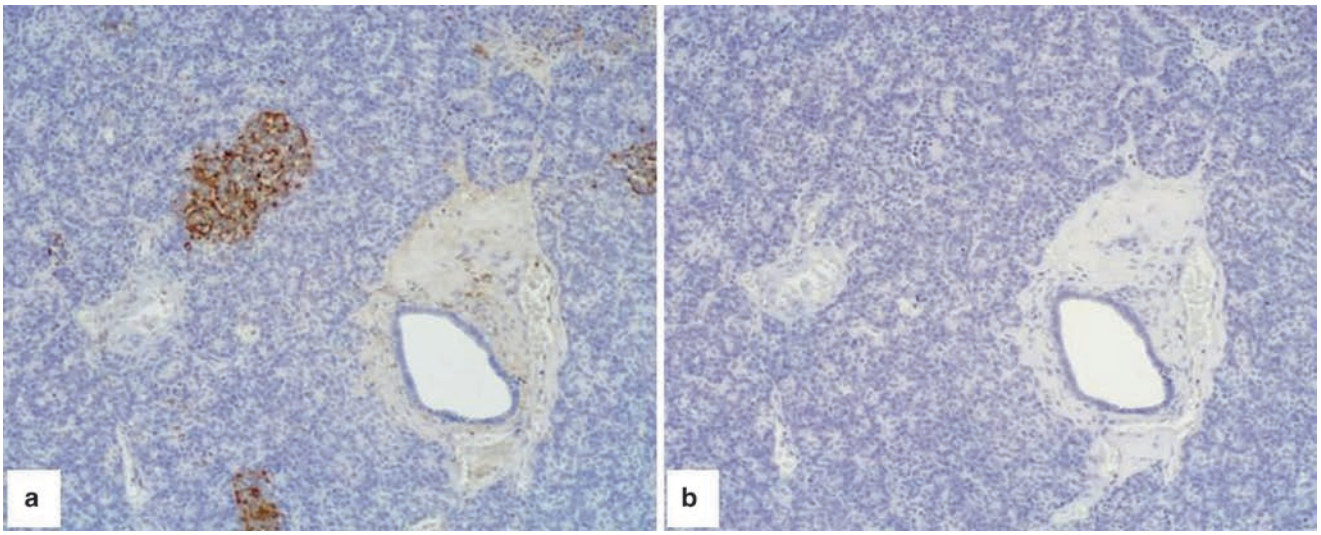

Fig. 17.4 Late phase of type 1 diabetes mellitus. The islets are mainly composed of glucagon-positive α-cells (**a**), while they completely lack β-cells as shown by negative staining for insulin (**b**)

destroy β-cells by activating apoptotic mechanisms [120]. In recent-onset type 1 diabetes, the insulitis is associated with increased expression of class I MCH and class II MHC molecules in a minority of β-cells [124]. 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 years [121]. 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 non-diabetic controls and the mean size of the islets is decreased [125]. β-cells are virtually absent or consistently reduced. The regular islets are mainly composed of α- and δ-cells (Fig. 17.4) while the irregular islets of the dorsal part of the head are composed of PP-cells [115, 126]. Although islet amyloidosis is normally absent in type 1 diabetic islets [113], islet calcification and fibrosis have been described [127]. The exocrine parenchyma displays acinar cell atrophy and some mild interstitial fibrosis [113, 116].

17.2.3 Type 2 Diabetes Mellitus

Type 2 diabetes mellitus accounts for over 90% of cases of diabetes [109] and afflicts individuals with insulin resistance, who generally have a relative rather than an absolute insulin deficiency. Patients are usually adults over the age of 40 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 [110].

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 about 10–20 years after the diagnosis of diabetes, the majority of pancreases examined at autopsy are from old patients with long-standing 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% [128]. The characteristic islet alteration in older patients with long-standing type 2 diabetes is amyloidosis [129], formerly called "hyalinization" [130]. Islet amyloid is composed of an amorphous acellular material that appears between the islet cells and the intrainsular capillaries but in advanced stage may also form globules that replace the islet cells. Typically, islet amyloid derives from amylin (also known as islet amyloid polypeptide-IAPP) deposition [131]. IAPP, a 37-amino acid peptide showing a close relationship with the calcitonin gene-related peptide [132], is synthesized by β-cells and co-stored in secretory granules together with insulin [133]. It is not clear whether islet amyloid represents a primary or secondary event in the pathogenesis of type 2 diabetes but, anyhow, it negatively interferes with islet cell function. Despite considerable studies during the past decade, mechanisms leading to islet amyloid formation are still not completely understood.

Recent data suggest a possible role of BACE2 protease in the formation and deposition of islet amyloid [134]. BACE2 is a close homolog of the aspartic protease BACE1 and both enzymes are involved in the formation of amyloid plaques observed in the brain of patients with Alzheimer's disease and in muscles of patients with sporadic inclusion-body myositis and hereditary inclusion-body myopathy. The role of these proteases in the formation of amyloid plaques depends on the cleavage of amyloid precursor protein (APP). BACE2 has been identified in secretory granules of rat and mouse β-cells and it has been thought to participate in the cleavage of IAPP protein and, probably, of APP which has been also recently identified in normal islet cells [135]. Amyloid, which appears as an eosinophilic amorphous deposition in 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. 17.5).

17.3 Proliferative Pathology

Proliferative pathology of the endocrine pancreas includes three disorders: islet hyperplasia, nesidioblastosis, and islet dysplasia [136]. Although islet hyperplasia and nesidioblastosis may coexist in the same pancreas, the two lesions can be observed independently.

17.3.1 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 over 250 μm in diameter (normally it is about 225 μm).

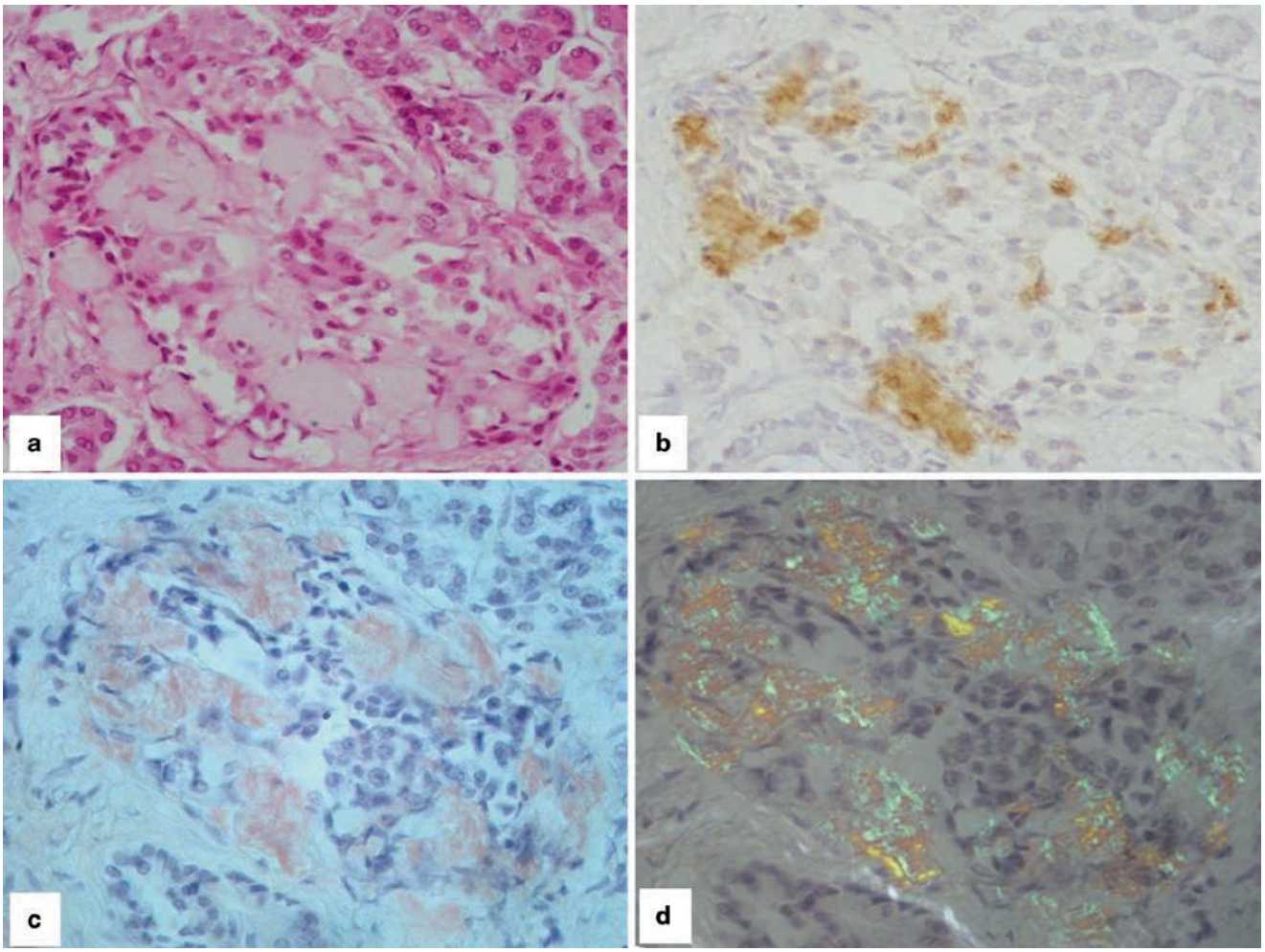

Fig. 17.5 Pancreatic tissue from an old patient with long-standing type 2 diabetes. H&E stain shows amorphous globules of amyloid that replace islet cells (**a**). Islet amyloid, which derives from amylin as demonstrated by immunohistochemical staining with anti-amylin antibody (**b**), is stained in *red* using Congo Red stain (**c**) and shows the typical *green* birefringence in polarized light (**d**)

Islet hyperplasia has been sporadically reported in asymptomatic subjects, in patients with alpha-1-antitrypsin deficiency [137] or associated with hyperfunctional syndromes such as hyperinsulinism, Zollinger–Ellison and Verner–Morrison syndromes [138–140]. However, the association between islet hyperplasia and either Zollinger–Ellison or Verner–Morrison syndrome has been questioned since no gastrin or VIP (vasoactive intestinal peptide) has been demonstrated in such hyperplastic islets [141, 142]. Islet hyperplasia has also been reported in neonates with maternal diabetes, erythroblastosis fetalis, acquired immunodeficiency syndrome (AIDS) [143] or with complex genetic or malformative syndromes such as Simpson–Golabi–Behmel syndrome [144], hereditary tyrosinemia of hepatorenal type [145], Zellweger's cerebro-hepato-renal syndrome [146], leprechaunism [147] and Beckwith–Wiedemann syndrome [148].

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 [136].

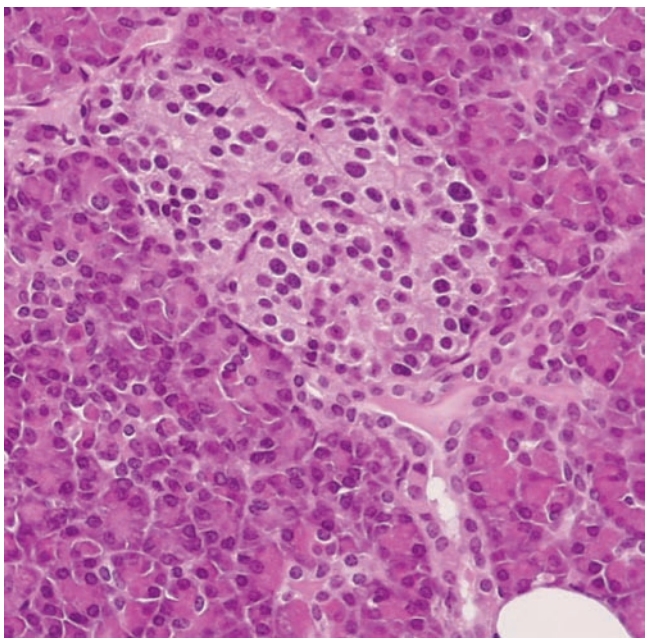

Fig. 17.6 Example of pancreatic nesidioblastosis. The islet of Langerhans is connected with a pancreatic ductule

17.3.2 Nesidioblastosis

The term nesidioblastosis was coined to designate endocrine clusters connected with pancreatic ductules (Fig. 17.6) indicating insular neogenesis from ductular cells [149]. This lesion unassociated 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.

17.3.2.1 Persistent Hyperinsulinemic Hypoglycemia in Infancy

Persistent hyperinsulinemic hypoglycemia in infancy (PHHI) is the most important form of congenital hyperinsulinism [150, 151]. 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 [150]. Therapeutic approaches include glucose infusions, diazoxide or octreotide therapy and, sometimes, subtotal pancreatectomy [152–154]. Physiologic and morphologic studies have recently indicated that PHHI is a hyperfunctional β-cell disorder associated with different pathologic changes [155]. In addition, molecular studies have demonstrated that PHHI results from at least three different genetic defects: (1) mutations in the two subunits SUR1 and KIR6.2 of the sulfonylurea receptor (SUR), encoded by *KNCJ11* and *ABCC8* genes, respectively [156–158]. 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. Mutations in *ABCC8* and *KCNJ11* genes result in K_{ATP} channels dysfunctions leading to continuous β-cell depolarization and inappropriate insulin secretion. More than 100 mutations have been described [159], and novel mutations are continuously identified [160]; (2) mutations of genes encoding for enzymes such as glucokinase and glutamate dehydrogenase which regulate the rate of insulin secretion [161, 162]; (3) loss of heterozygosity (LOH) at region 11p15.1 of maternal alleles unmasking paternally inherited recessive SUR1 or KIR6.2 mutations [153, 163, 164].

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) [165–168]. 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 [169].

Focal PHHI

Focal PHHI is found in a quarter to a half of all PHHI [92, 163] and is associated with paternally inherited *ABCC8* mutation (less frequently *KCNJ11*) and with specific loss, restricted to the lesion, of maternal alleles of the 11p15 region [163]. Macroscopically, the pancreas has a normal appearance. Usually the lesion is unifocal and it has been located either in the head and body [153] or in the body and tail of the pancreas [155]. 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 of the four islet cell types, however β-cells are more numerous than in normal islets, representing 70–90% of all endocrine cells [155]. 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. From a diagnostic point of view, it is worth noting that focal PHHI is a non-neoplastic disease and it must not be considered as an insulinoma. This is supported by both morphological and molecular features [170].

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 [171], prominent ductulo-insular complexes, abundant poorly defined endocrine cell clusters (some large) with often irregular outlines, and islets of variable size [155, 165, 166, 172, 173]. 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 [167, 174]. 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 [175]. 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 [165]. Although a reduced number of α- and δ-cells has been reported in some investigations [174, 176], this finding has not been confirmed in other studies [166].

17.3.2.2 Persistent Hyperinsulinemic Hypoglycemia in Adult

Persistent hyperinsulinemic hypoglycemia in adult (PHHA) is a very rare cause of persistent hyperinsulinemia and hypoglycemia not associated with insulinoma. About 55 cases have been reported in the world literature [177, 178]. 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 years 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 of the ABCC8, KCNJ11 or glucokinase genes have not been identified. The cause of PHHA is not known, but the recently observed association with gastric by-pass surgery in obese patients suggests that a reactive process possibly unmasks or induces a defect in β-cells, resulting in hyperfunction [178].

Histologically, the most characteristic finding is represented by the presence of hypertrophic β-cells showing large hyperchromatic nuclei and abundant cytoplasm, while α-, δ-, and PP cells do not show significant cytologic abnormalities. Interestingly, the hyperfunctional state of β-cells is not associated with changes in the subcellular distribution of insulin and proinsulin, proliferative activity and mutation of the menin gene [179]. Hypertrophic β-cells are scattered as single elements or grouped in small clusters (Fig. 17.7) 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 [136].

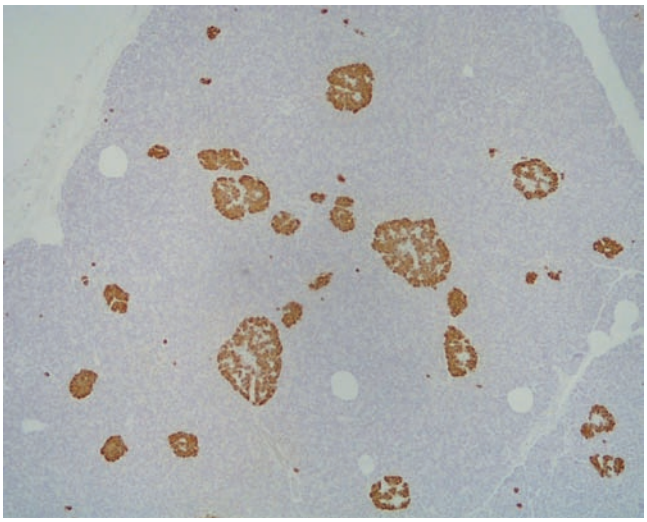

Fig. 17.7 Pancreatic tissue from an adult patient with PHHA. β-cells positive for insulin are present both in the islets and as single elements or small clusters throughout the exocrine parenchyma

17.3.3 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 MEN-1 syndrome [180]. 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 [181–184].

17.4 Endocrine Tumors of the Pancreas

17.4.1 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 pre-existing differentiated exocrine and endocrine cells or from regenerating ductules which give origin to new pancreatic lobules and islets [185]. Interestingly, insulin-like growth factor-1 (IGF-1) is abundantly expressed in the loose connective matrix surrounding the newly formed ductules [186].

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 [187–189]. 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, non-dysplastic 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 [190].

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 [191]. In MEN1 pancreas, moreover, multiple lesions associated with tumors point to a complex multistep process involving well differentiated islet cell types [192, 193]. All subsequent steps in tumorigenesis found in human MEN1 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 MEN1 tumor suppressor mouse knockout model [194]. However, in human MEN1 patients loss of one MEN1 allele only has been recently demonstrated in microadenomas and in small monohormonal endocrine cell clusters within the islets, while it was conversely lacking in normal islets and hyperplastic and enlarged islets with an increased number of glucagon cells. This interesting finding suggests that islet hyperplasia is not an obligatory step in human pancreatic MEN1-associated tumor development [195].

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 [196], several investigators have tried to elucidate the clinico-pathological and molecular characteristics of these neoplasms. Because different methodological approaches have been used to classify these tumors, a variety of nomenclatures have been proposed and they have often created confusion among pathologists and clinicians [197]. In 1995, a group of endocrine pathologists [198] 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 [199–202]. The first report indicating the utility of a proliferative marker in predicting the malignancy of PETs was published in 1992 by Pelosi et al. [203]. These authors demonstrated that a PCNA index higher than 5% correlated with a decreased mean survival of patients. However, the Ki67 index, evaluated using the monoclonal antibody MIB1, has emerged from different studies to be better than the PCNA proliferative rate in predicting patient outcome [204–211]. In recent years several studies [204–217], with only a few exceptions [218, 219], have demonstrated the useful role of Ki67 proliferative index as prognostic indicator of long term outcome in PETs (Fig. 17.8). In addition, the usefulness of Ki67 stain in cytological smears for pre-operative evaluation of pancreatic endocrine tumors has been also suggested [220]. However, in addition to the Ki67 index, a number of clinicopathological parameters have been investigated and proved to be useful as behavior-predicting variables for patients with endocrine tumors of the pancreas [193, 204]. The following clinicopathological criteria of malignancy should be considered: presence and type of the endocrine syndrome, 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 reduced nuclear/cytoplasmic ratio, more than two mitoses per 10 HPF, perineural invasion, vascular invasion, and presence of bands forming fibrosis. Among these criteria mitotic rate, neuroinvasion with or without vascular invasion, peritumoral or stromal infiltrative growth, tumor size and endocrine syndrome other than insulinomas have been recently proved to be effective in predicting recurrence and disease-specific death among well differentiated PETs [217]. In addition, cytokeratin 19 expression has been recently proposed as a powerful independent prognostic marker

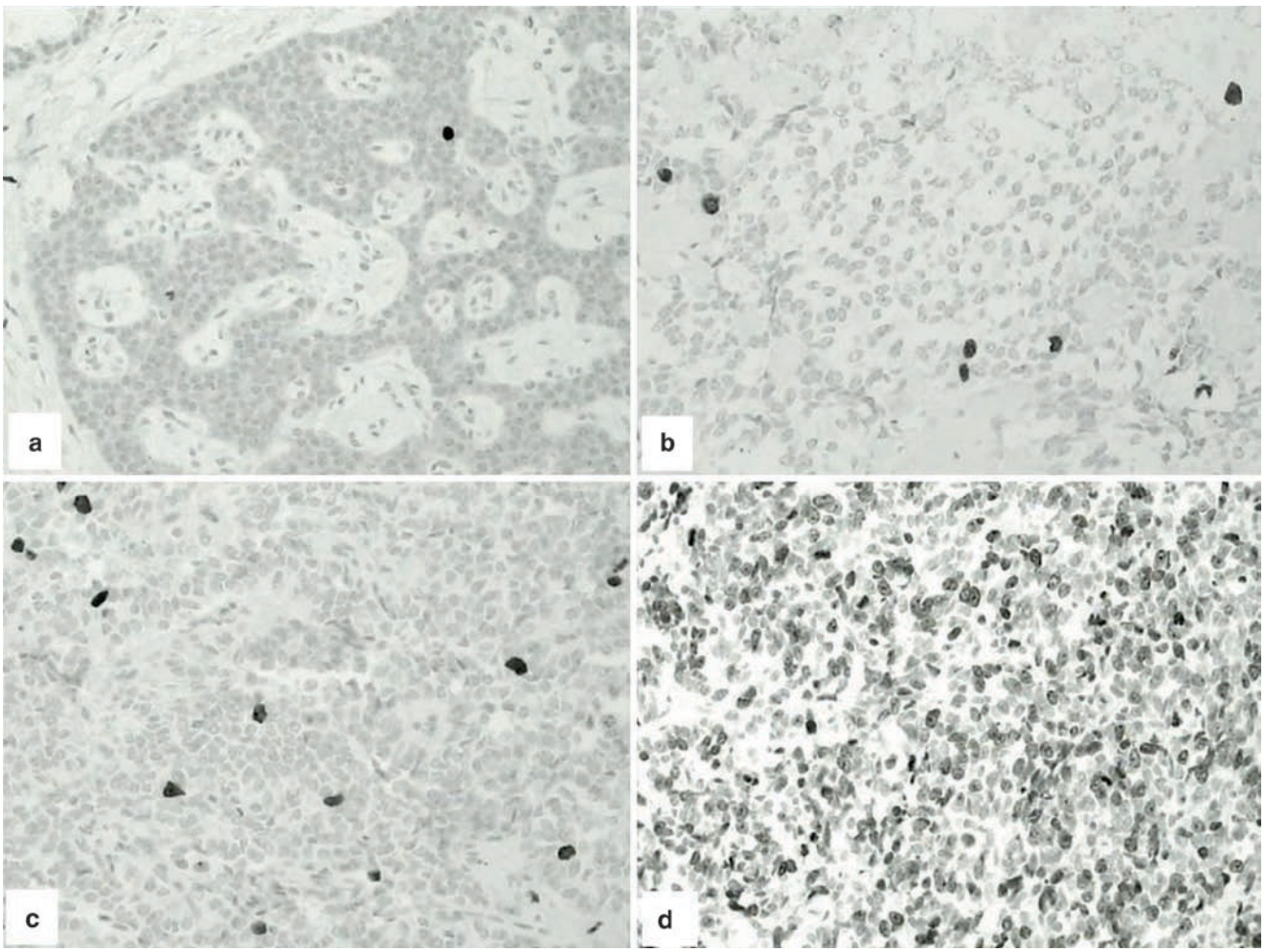

Fig. 17.8 Ki67 immunoreactivity in different types of pancreatic endocrine tumors. The Ki67 proliferative index increases progressively from benign tumors (**a**) to poorly differentiated endocrine carcinomas (**d**). Tumors with uncertain behavior (**b**) and well differentiated endocrine carcinomas (**c**) show an intermediated Ki67 proliferative index

[212, 221, 222], although according to one study [223] it did not appear as a powerful prognostic indicator, especially if compared with Ki67 index. The clinical usefulness of other potential markers of malignancy such as topoisomerase IIα [224], cyclooxygenase (COX)-2 [225], nuclear survivin [226], lymphatic vessel density, and vascular endothelial growth factor-C (VEGF-C) expression [227] remains to be confirmed in studies based on large trials. The criteria proposed by the international group of pathologists in 1995, together with the various above mentioned, more reliable, prognostic parameters identified in the last years, represented the basis for the WHO classifications [228, 229] of pancreatic endocrine tumors (Table 17.5) The practical value of the WHO classifications and its use in routine diagnostic approach to PETs have now been confirmed in several studies [212–214, 217, 230–233] In addition to the WHO classifications, a TNM staging system and a proliferative grading system have been recently proposed [234] to stratify PETs in relation to their extension (Table 17.6). This staging approach seems to have also a prognostic value as confirmed by recently published investigations [217, 232, 233].

Endocrine tumors of the pancreas are classified according to the tumor cell type or to 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 tumor itself may be denominated according to the associated syndrome as "insulinoma," "gastrinoma," etc. Remarkably, the tumor-associated hyperfunctional syndrome is per se more indicative of the tumor behavior than the morphologic cell typing (Table 17.7).

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

Table 17.5 WHO classification of pancreatic endocrine tumors [228].

1. *Well differentiated endocrine tumor*
   - 1.1. *Bening behavior*: confined to the pancreas, nonangioinvasive, <2 cm in size; ≤2 mitoses × 10 HPF and ≤ 2% Ki67 positive cells
     - 1.1.1. Functioning: insulinoma
     - 1.1.2. Nonfunctioning
   - 1.2. *Uncertain behavior:* confined to the pancreas, ≥2 cm in size, >2 mitoses × 10 HPF and > 2% Ki67 positive cells, 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, etc.

Table 17.6 TNM staging system of pancreatic endocrine tumors [234].

T-primary tumor	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Tumor limited to the pancreas and size <2 cm
T2	Tumor limited to the pancreas and size 2–4 cm
T3	Tumor limited to the pancreas and size > 4 cm or invading duodenum or bile duct
T4	Tumor invading adjacent organs (stomach, spleen, colon, adrenal gland) or the wall of large vessels (celiac axis or superior mesenteric artery)
	For any T, add (m) for multiple tumors
N-regional lymph nodes	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis
M-distant metastases	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Stage I			
Stage I	T1	N0	M0
Stage IIa	T2	N0	M0
Stage IIb	T3	N0	M0
Stage IIIa	T4	N0	M0
Stage IIIb	Any T	N1	M0
Stage IV	Any T	Any N	M1

Table 17.7 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, etc.	50–90% malignant low grade
20	Nonfunctioning, locally symptomatic tumors	70–80% malignant low grade
1–5	Small cell carcinoma with poor endocrine differentiation	All malignant, high grade

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 non-functioning 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, low mitotic and proliferative status together with the frequently observed trabecular structure. As a rule, well differentiated PETs confined to the pancreas that are non-angioinvasive, show ≤ 2 mitoses per 10 HPF, ≤2% Ki67 positive cells, and are less than 2 cm in diameter follow a favourable course. Well differentiated PETs confined to the pancreas but showing angioinvasion and/or perineural invasion, or >2 mitoses/10 HPF, or >2% Ki67 positive cells are at increased risk for malignant behavior (uncertain behavior). *Well differentiated endocrine carcinomas (WDECs)* are epithelial growths which 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 (2–9/10 HPF) or Ki67 proliferative index (2–10%). Other important diagnostic features are perineural invasion, angioinvasion and bands forming fibrosis [217] (Fig. 17.9). In addition to conventional histological features, tumors composed of cells showing clear

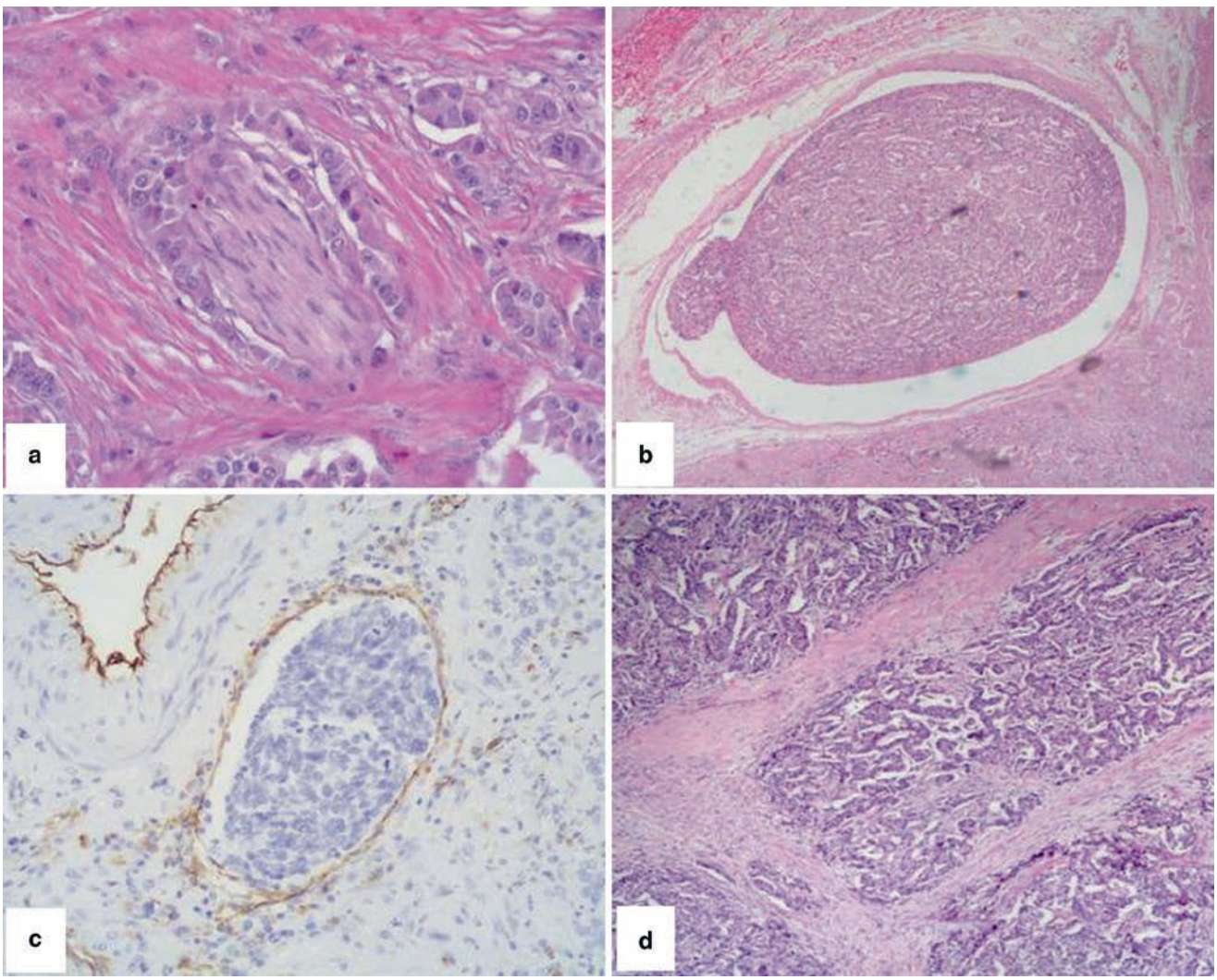

Fig. 17.9 Neuroinvasion (**a**), vascular invasion of both small venules (**b**) and capillaries (**c**), and the presence of stromal fibrous bands dissecting tumour parenchyma (**d**) have been demonstrated to be good predictors of malignancy. In (**c**), the endothelial cells are stained using an anti-CD31 antibody

lipid-rich or strongly eosinophilic (oncocytic-type) cytoplasms [235, 236] or cells with marked nuclear atypia (pleomorphic tumors) [237] have been described. *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 (small cell variant of PDEC) and, in some cases, large in size (large cell variant of PDEC), showing more than 20 mitoses/10 HPF, more than 20% Ki67 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. The possibility of an intermediate category with prognostic significance between well and poorly differentiated pancreatic endocrine carcinomas has emerged in the last years. It is a quite common experience among pathologists and clinicians expert in pancreatic pathology that there are tumors lacking the typical histological features of the small cell or large cell variants of PDECs, but showing a behavior worse than that of WDECs. A similar category of tumors has been also identified in the stomach [238]. Criteria for identifying such category of "moderately differentiated endocrine carcinomas" are still matter of debate: in our experience a mitotic index between 10 and 20 mitoses per 10 HPF, focal necrosis in addition to infiltrative histological features including neuroinvasion and angioinvasion are the most useful [217]. The mean survival of patients bearing this tumor type is 28 months, which is not statistically different from the survival of patients with PDECs, but is significantly different from that of patients with WDECs. Some authors have recently proposed a simple classification scheme alternative to the multiparametric approach of the WHO classifications. They considered all PETs as potentially malignant and they divided well differentiated PETs into low and intermediate grade groups on the basis of tumor necrosis and of a mitotic rate from more than 2–10 mitoses per 50 HPF [218, 239, 240]. This simplified approach has identified two prognostically different groups. However, in our experience although the intermediate group could represent a well defined category, the low grade group comprises a heterogeneous group of PETs with a different outcome and risk of recurrence. [217].

In addition to traditional radiological investigations including abdominal ultrasonography and computed tomography, octreotide scintigraphy has emerged as a useful tool for the diagnosis of PETs. The biological basis is represented by the fact that PETs, unlike exocrine pancreatic neoplasms, express somatostatin receptors [102] and there is a high concordance between somatostatin receptor expression at tissue level and ^{111}In-pentetreotide scintigraphy [103]. Somatostatin receptor type 2 is the more expressed receptor in both normal islet cells and PETs, with the exception of insulinomas that mainly express the subtype 5. It has been recently demonstrated that the immunohistochemical expression of somatostatin receptor in tumor tissues has a good agreement with in vivo scintigraphy only when membrane stainings are considered. Conversely, cytoplasmic positivity shows poor correlation with somatostatin receptor scintigraphy [103].

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 alpha-1-antitrypsin favors a solid-pseudopapillary neoplasm [193]. It is worth recalling that NSE reactivity also occurs in solid-pseudopapillary tumors so that it cannot be used as differential diagnostic marker [241]. 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, PAS-positive granules, positive immunostaining for the COOH-terminal portion of BCL10, carboxyl ester hydrolase, trypsin, lipase, and the lack of immunoreactivity for chromogranins, synaptophysin and hormones strongly favor the diagnosis of acinar cell carcinoma [193, 242]. Age represents an important criterion in distinguishing WDETs from pancreatoblastomas since the former are very rare in the first decade of life. Anyhow, the presence of squamoid nests, an acinar structure, and reactivity for alpha-fetoprotein supports the diagnosis of pancreatoblastoma [241, 243].

17.4.2 Well Differentiated Functioning Endocrine Tumors

17.4.2.1 Insulinoma

Insulinoma is the most common type of functioning PET [193, 240, 244–246] and the incidence has been estimated to be $1/1.25 \times 10^6$ persons [247]. Although insulinomas can occur at any age, they are more frequent between 30 and 60 years of age. Children under 15 are rarely affected [248, 249]. Up to 90% of insulinomas are benign solitary tumors suitable for surgical resection or enucleation [250] whereas malignant tumors are present in only 5–10% of cases [251]. 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 [252].

Insulinomas occur in any part of the pancreas [253] but they are more frequent in the body–tail region [193].

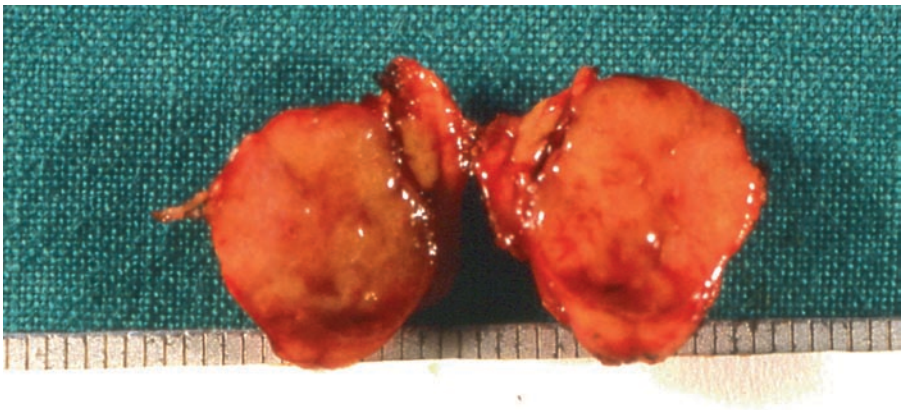

Fig. 17.10 Gross appearance of an insulinoma of the pancreatic body. The tumor was enucleated and is well circumscribed

The tumor is single in the majority of cases although multiple nodules may coexist, a finding that must suggest the presence of a MEN-1 syndrome [193, 254]. 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. 17.10).

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. 17.11). Such deposits show the typical green birefringence after staining with Congo Red and examination in polarized light (Fig. 17.12). As in the islets of type 2 diabetic patients, the amyloid of insulinomas contains amylin (IAPP) [255]. Tumor nuclei in cases with benign behavior are round to ovoid with finely stippled chromatin and inconspicuous nucleoli. Immunohistochemically, almost all insulinomas are positive for insulin and proinsulin as well as for amylin [256, 257] (Fig. 17.12). About half of insulinomas are multihormonal and cells positive for glucagon, somatostatin, PP, gastrin, ACTH, and calcitonin have been described [256–258]. In addition to hormone peptides, insulinomas express the pro-hormone convertases PC2 and PC3, the carboxypeptidase H (CPH) and 7B2, which play a role in the proteolytic conversion of proinsulin to insulin [259]. Malignant insulinomas have been found to be immunohistochemically positive for TGFα, K-ras, N-ras, and p53, and point mutations at codon 12 of K-ras gene have been also demonstrated [260].

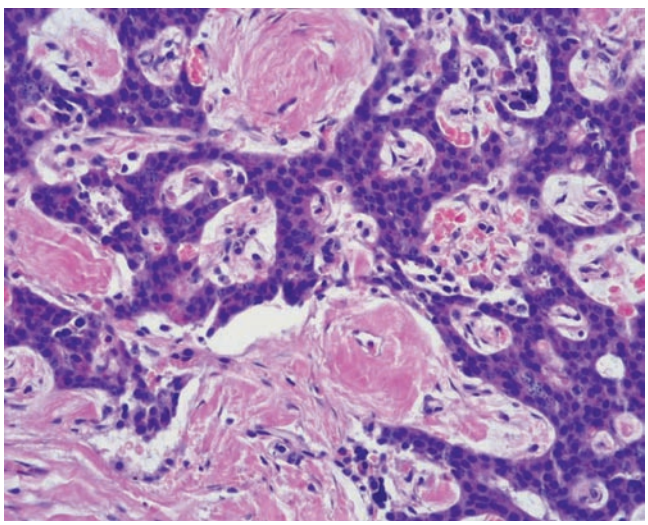

Fig. 17.11 Microscopic feature of the insulinoma shown in Fig. 17.10. Cells are uniform with no or mild atypia and form trabecular structures separated by amorphous globules of amyloid

Ultrastructurally, three main types of cells have been described: (1) densely granulated cells with typical crystalline-type beta-granules (Fig. 17.13); (2) cells with solid, round to slightly pleomorphic granules, and (3) sparsely granulated cells [261, 262]. According to Berger et al. [261] densely granulated tumors (type A) are functionally characterized by a good response to diazoxide and somatostatin 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 years.

17.4.2.2 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 [252]. Glucagonomas are rare tumors representing about 8% of functioning neoplasms and 5% of all clinically relevant pancreatic endocrine tumors [193, 240, 263], with an annual

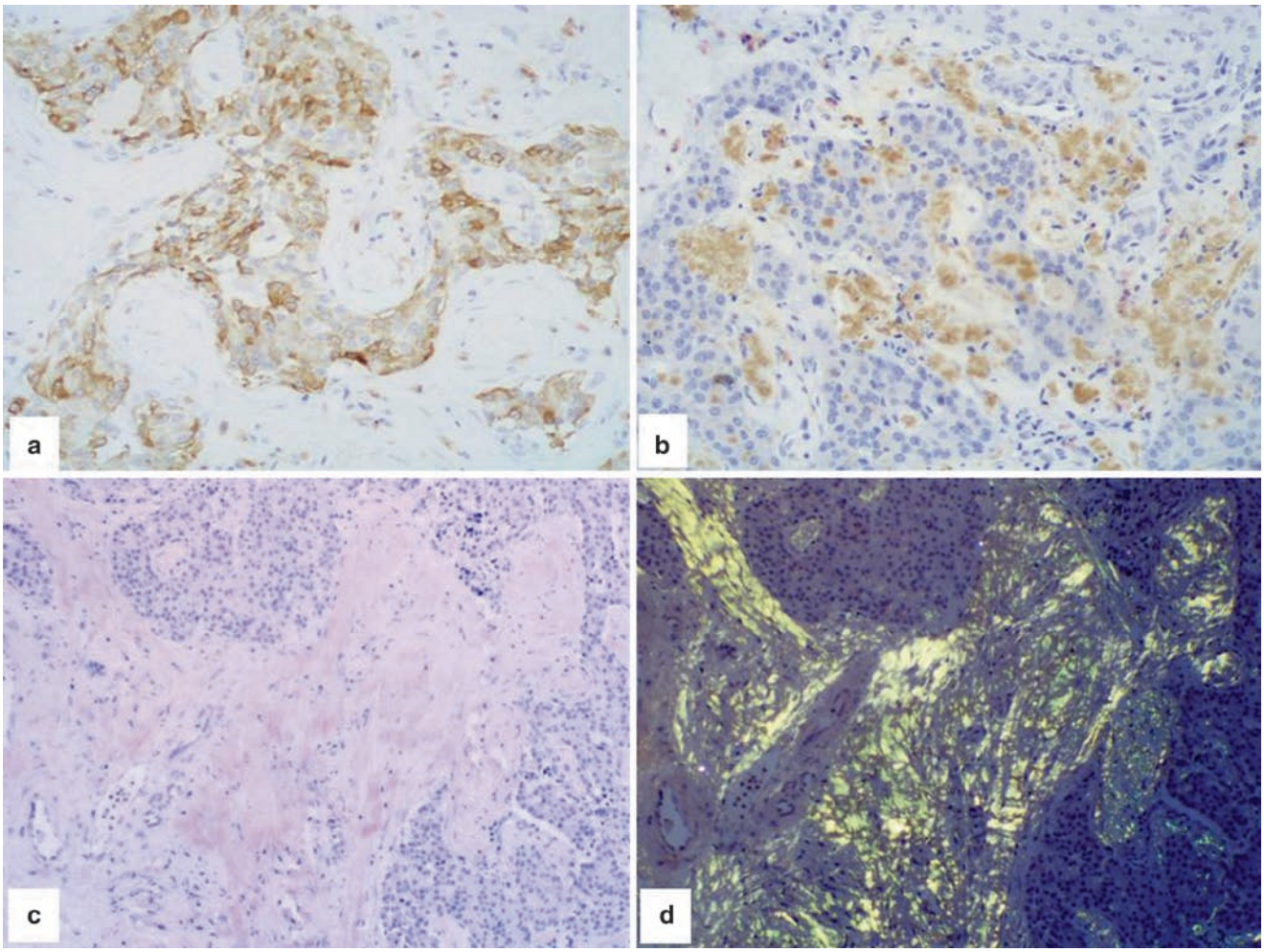

Fig. 17.12 Insulinoma. Insulin-immunoreactivity is localized in neoplastic cells (**a**), whereas amyloid depositions, which are completely insulin negative, are immunoreactive for IAPP (**b**). Amyloid is stained in red with Congo Red stain (**c**) and shows the typical green birefringence in polarized light (**d**)

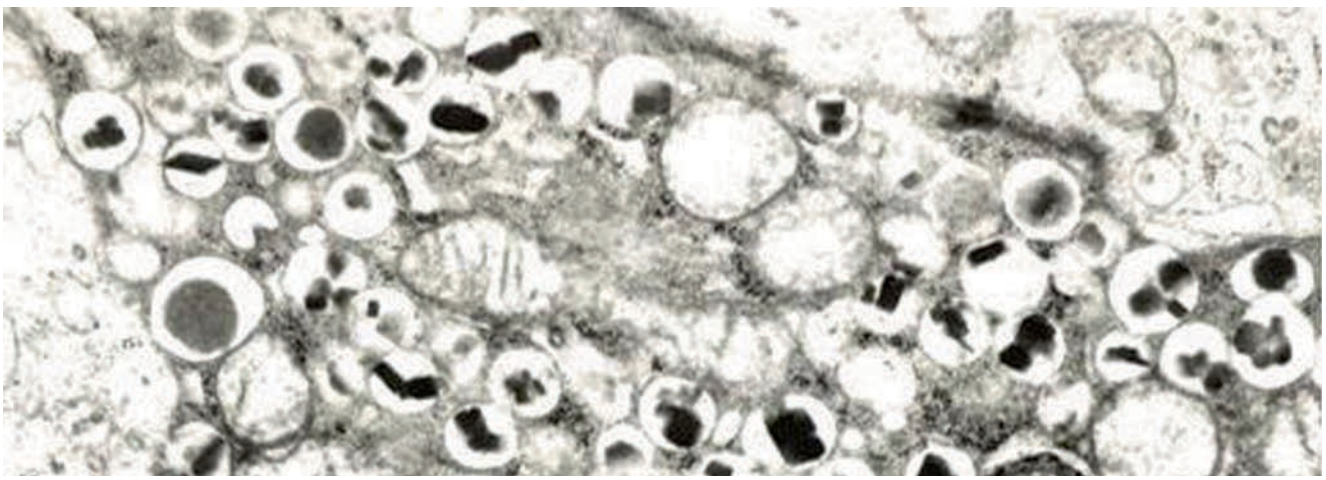

Fig. 17.13 Ultrastructural features of a pancreatic insulinoma. Tumor cells contain numerous typical crystalline type secretory granules (**a**); (**b**) is an example of immunogold technique showing that amyloid deposits are positive for amylin

Table 17.8 Incidence and malignancy rates of different types of well differentiated pancreatic endocrine tumors (modified from [270]).

Tumor type	Incidence (new cases per 10^6 population/year)	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%

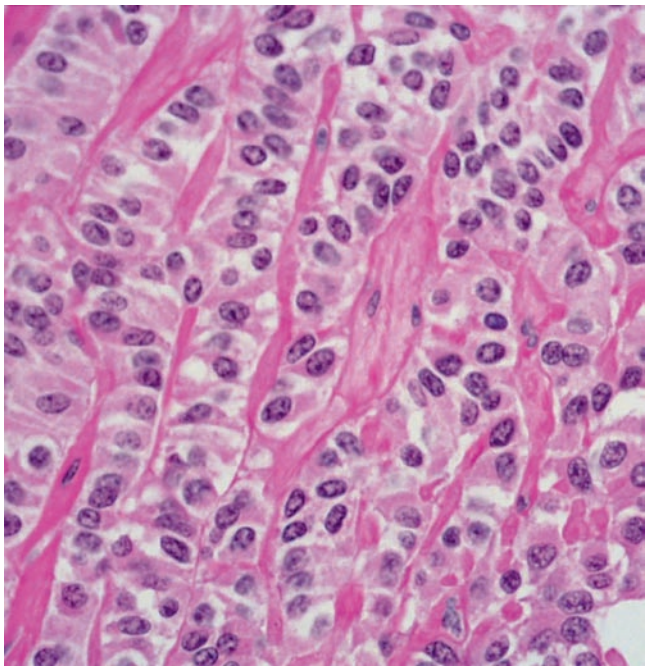

Fig. 17.14 Glucagonoma of the pancreas. Tumor cells are well differentiated and form trabecular structures

incidence of 0.01–0.1 cases per 10^6 population (Table 17.8). They occur most often in adult patients (average age of 55 years) and are slightly more common in women. Glucagonomas must be distinguished from small nonfunctioning glucagon-producing tumors which are often incidentally found at autopsy or at surgery and frequently have 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. 17.14). Tumor cells are polygonal with faintly granular, often abundant, cytoplasm. Vascular and perineural invasion is frequently observed; mitoses and nuclear atypia are rare. Tumor cells are strongly stained with the Grimelius' silver impregnation.

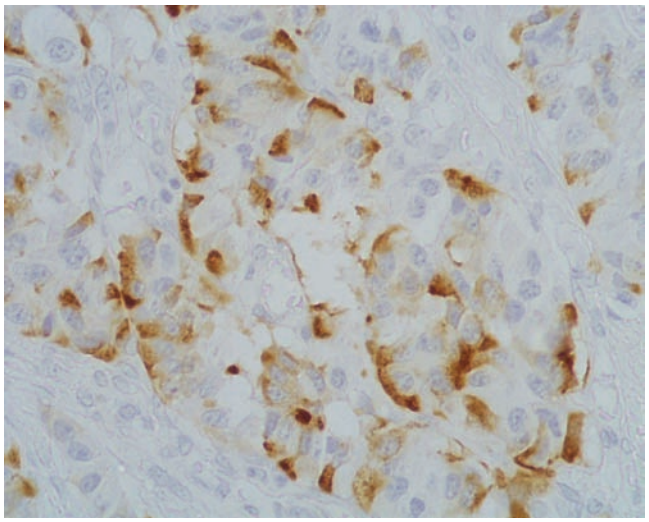

Fig. 17.15 Glucagon immunoreactivity in a pancreatic glucagonoma

Immunohistochemistry reveals positivity for glucagon (Fig. 17.15) and for one or more peptides derived from proglucagon, such as glicentin and glucagon-like peptides (GLP)-1 and GLP-2 [261, 267]. In addition, glucagonomas may also contain PP-, δ-, and β-cells as revealed by pancreatic polypeptide, somatostatin, and insulin immunoreactivities [193, 266].

At the ultrastructural level, three types of secretory granules have been identified in glucagonomas [268, 269]: (1) typical A-cell granules (Table 17.3) (Fig. 17.16); (2) "atypical" or "unspecific" small to medium-sized round granules with a uniform core of varying density; (3) medium-sized granules resembling alpha-granules of the fetal islets.

Approximately 80% of glucagonomas are malignant and 70% are metastatic at the time of diagnosis [193, 240]. 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]. It has been recently demonstrated that patients with glucagonomas respond better than the general group of malignant PETs to treatment with streptozotocin and 5FU [263]. 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 glucagonoma developed a second, or even third, hyperfunctional syndrome, including the Zollinger–Ellison and hyperinsulinemic syndrome [271].

17.4.2.3 Somatostatinoma

Pancreatic somatostatinoma is an uncommon endocrine tumor composed of δ-cells, associated with a complex of symptoms (the somatostatinoma syndrome) caused by

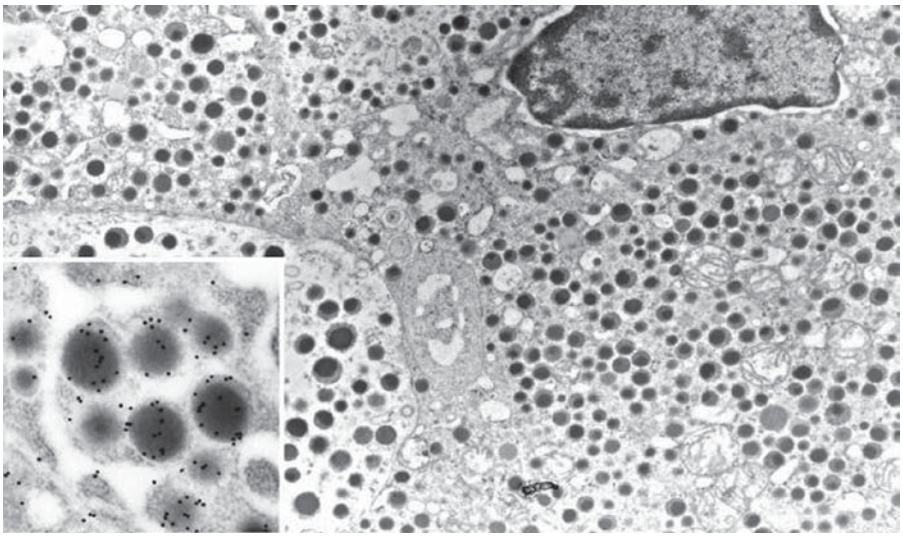

Fig. 17.16 Ultrastructural aspect of a pancreatic glucagonoma. Tumor cells are rich in typical α-cell granules with a central or eccentrical electron-dense core surrounded by a *pale halo*. Glucagon immunoreactivity is restricted to secretory granules (inset)

Table 17.9 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 (30–74)	56	12	32	6.3 (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

hypersomatostatinemia. As yet only a small number of pancreatic somatostatinomas (about 40 cases) have been described [272–284] and their incidence is considered to be less than 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 [285]. Pancreatic somatostatinomas prevail in females (Table 17.9) and arise in adults, with an average age at diagnosis of 55 years (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. 17.17). 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 [284]. Amyloid deposits are infrequent [274, 276] and, unlike those of insulinomas, do not react with anti-amylin antibodies [276]. Tumor cells show varying degree of immunoreactivity for somatostatin (Fig. 17.17). In addition, several cases show positivity for other peptides, including calcitonin, adrenocorticotropin and gastrin [193, 286].

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 clinicopathological differences which 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. 17.17), do not induce the classical somatostatinoma syndrome (nonfunctioning tumors), and show a relatively strong association with the von Recklinghausen disease (neurofibromatosis type 1) [287, 288]. Somatostatin-producing endocrine tumors of both duodenum and pancreas have been also found in patients with MEN1 syndrome [285].

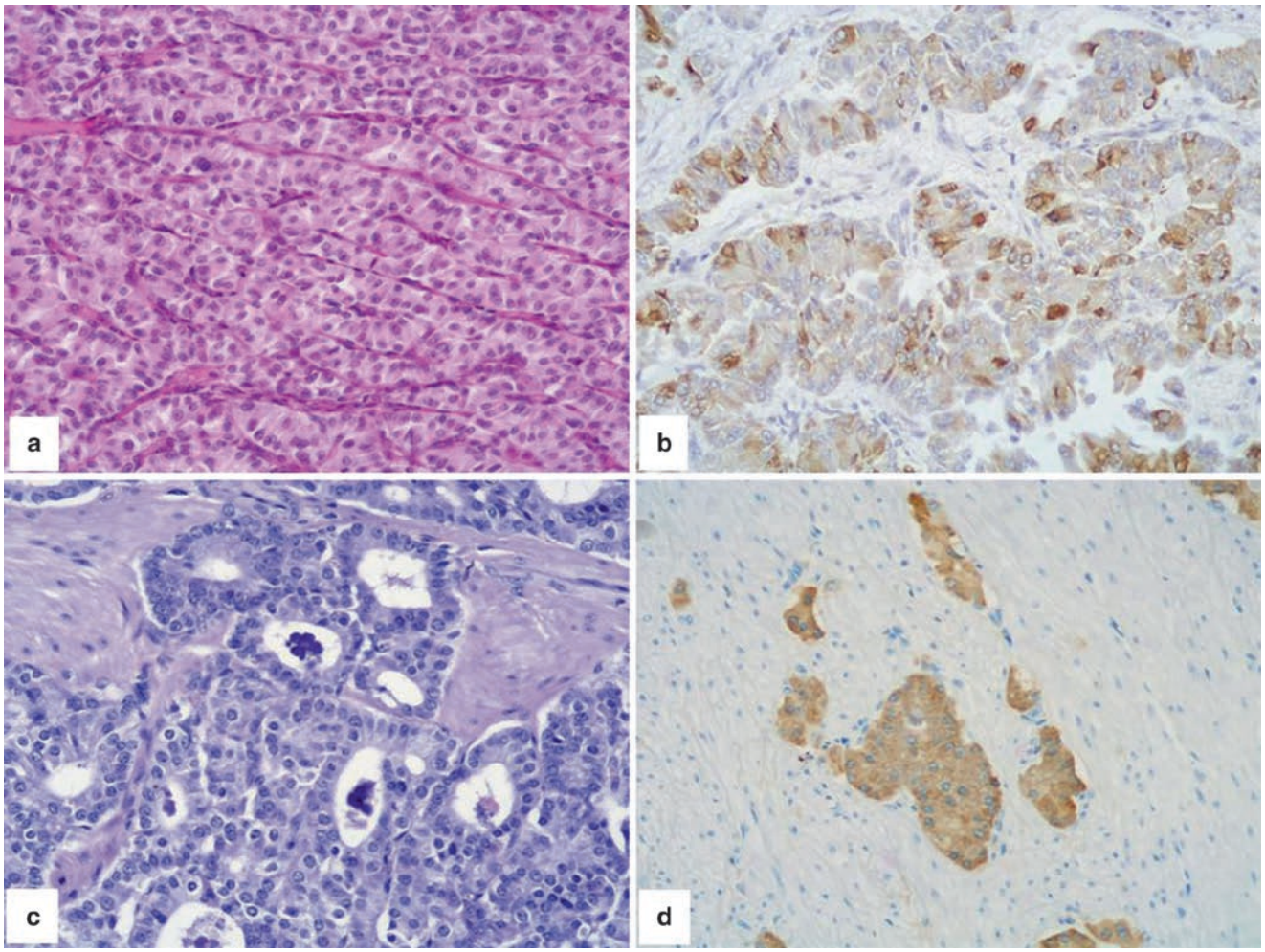

Fig. 17.17 δ-cell tumors of the pancreas (**a**, **b**) and duodenum (**c**, **d**). Pancreatic tumor shows a trabecular architecture (**a**), while the duodenal neoplasm presents the typical acinar pattern with psammomas. **b** and **d** are examples of immunostaining for somatostatin

17.4.2.4 VIPoma

VIPoma is an endocrine tumor, predominantly occurring in the pancreas, which produces the Verner–Morrison syndrome (WDHA: watery diarrhea, hypokalemia, achlorhydria) due to the secretion of vasoactive intestinal peptide (VIP), peptide histidine methionine (PHM), and other hormone-like substances. Pancreatic VIPomas are rare neoplasms accounting for about 5–8% of all PETs [197, 246]. VIPomas of the pancreas are the most frequent neoplasms associated with the WDHA syndrome [289]. 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 [289–291]. The average age of insurgence of pancreatic VIPomas is 50.5 years (range 15–82 years) and is much higher than the average age of patients with extrapancreatic neurogenic VIP-secreting tumors (average age 7.3 years). 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 [289].

The tumor is single in 95% of cases and is more frequently located in the tail of the gland [193, 289]. The diameter ranges from 1.5 to 20 cm with an average size of about 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 less than 2 cm, 50% for those with a diameter from 5 to 10 cm, and 71.4% for those larger than 10 cm (Table 17.10).

Histologically, pancreatic VIPomas show three main structural patterns: solid, trabecular, and tubulo-acinar (Fig. 17.18) in order of decreasing frequency [193]. 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 the cases, most of which have lymph nodes and/or liver metastases. The majority of neoplasms stain with Grimelius' method and are

Table 17.10 Clinicopathologic profile of pancreatic VIPomas and of non-pancreatic VIP-secreting tumors.

	Pancreatic	Non-pancreatic	*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 paper by Soga and Yakuwa [289]
Ne not evaluated; *NS* not significant

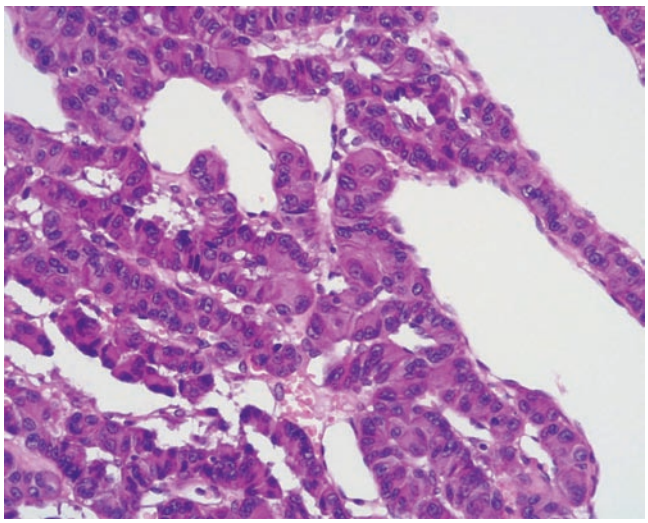

Fig. 17.18 Pancreatic VIPoma showing a characteristic tubulo-acinar pattern with cystic formations

immunoreactive for general endocrine markers and VIP (Fig. 17.19). 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 17.11). The frequent occurrence of PP-cells in pancreatic VIPomas and

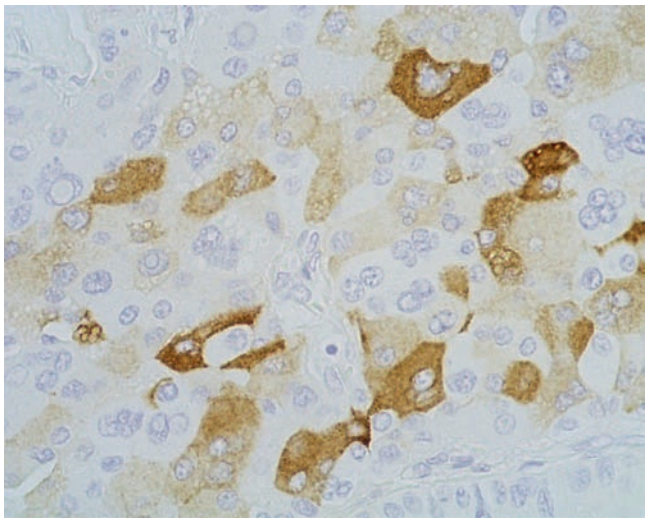

Fig. 17.19 VIP immunoreactivity in a pancreatic VIPoma

Table 17.11 Histochemical, immunohistochemical, and ultrastructural profile of pancreatic VIPomas and of non-pancreatic VIP-secreting tumors.

Marker	Pancreatic	Non-pancreatic	*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 paper by Soga and Yakuwa [289]
Ne not evaluated; *NS* not significant

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 [142].

Ultrastructurally, most tumors are composed of sparsely granulated (Fig. 17.20) 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 [193].

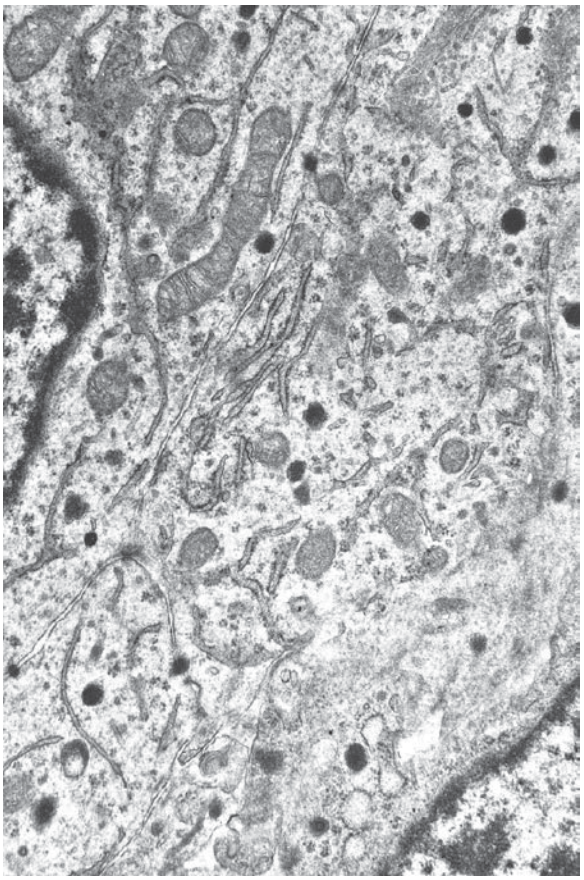

Fig. 17.20 Ultrastructural aspect of a pancreatic VIPoma. Tumor cells are sparsely granulated and secretory granules are small and thin-haloed with a moderately dense core

Up to 80% of VIPomas are reported to be metastatic at the time of diagnosis [289]. Surgery is the first choice of treatment, but in not resectable neoplasms chemotherapy and, especially, long acting somatostatin analogues may help in controlling the clinical endocrine symptoms [292].

17.4.2.5 Gastrinoma

Gastrinoma is an endocrine tumor, frequently malignant, which most often occurs in the pancreas although it can arise in extrapancreatic sites such as the duodenum, upper jejunum, and stomach [293]. 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, 294, 295]. 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 [193, 270]. There is a slightly male prevalence (male/female ratio: 3:2) and the mean age of insurgence is 38 years (range 7–83 years) [193, 240]. Evidence of MEN1 is found in 21% of patients with ZES [296].

Grossly, pancreatic gastrinomas are generally well circumscribed, but not encapsulated, tumors that are more frequently located in the head of the pancreas [193, 297].

Histologically, gastrinomas may present all architectural patterns classically observed in pancreatic endocrine tumors. However, the most common arrangement of cells is the formation of trabeculae. Tumor cells generally have round to ovoid rather uniform nuclei with slight to moderate atypia and mitoses are rarely found (Fig. 17.21). 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 like Grimelius' silver, chromogranins A and B, and synaptophysin. The definitive diagnosis is achieved by the 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. 17.21). In addition to gastrin, tumor cells may also show PP-, glucagon-, insulin-, somatostatin-, ACTH-, and serotonin immunoreactivities [298, 299], as well as positivity for exocrine markers including cytokeratin 19, carcinoembryonic antigen and epithelial membrane antigen [300].

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 [301, 302]. Interestingly, liver but not lymph nodes 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 [301, 302].

17.4.2.6 Enterochromaffin-Cell Tumors

Serotonin-producing (EC)-cell tumors of the pancreas associated with the classical carcinoid syndrome are rare, accounting for about 90 cases described in the world literature, and predominantly malignant (well differentiated endocrine carcinomas) [193, 303, 304]. Small, well differentiated, enterochromaffin (EC)-cell tumors non metastatic and lacking association with the carcinoid syndrome (with benign or uncertain behavior) have been also reported [193, 204, 240]. Histologically, the tumor is more frequently formed by solid nests and/or trabeculae (Fig. 17.22),

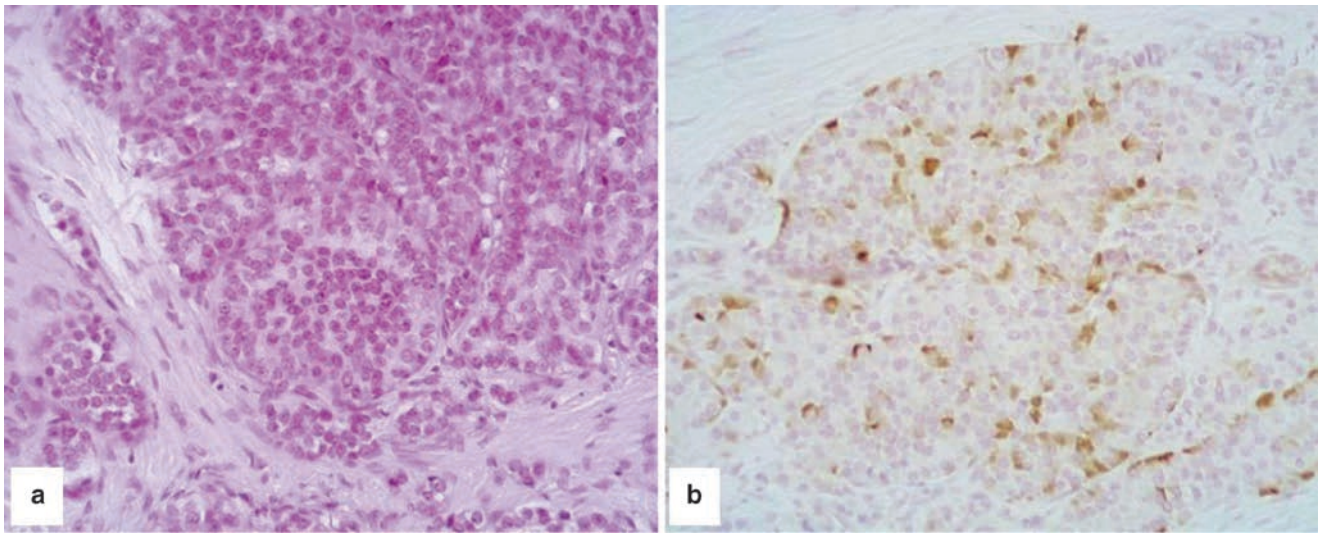

Fig. 17.21 Malignant pancreatic gastrinoma composed of moderately atypical well differentiated endocrine cells forming solid structures (**a**) and immunoreactive for gastrin (**b**)

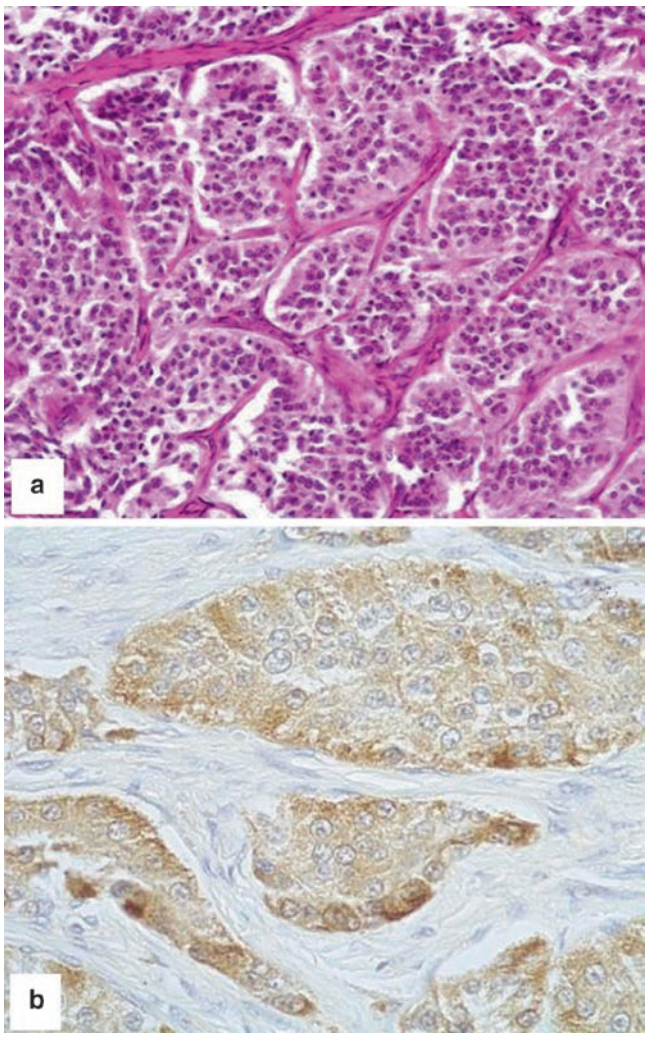

Fig. 17.22 EC-cell tumor of the pancreas composed of solid nests of well differentiated cells (**a**), which are positive for serotonin (**b**)

although a diffuse pattern with or without necrosis has been observed in large malignant tumors. Some tumors display a poorly differentiated aspect [305–307]. Well differentiated tumors (mostly nonfunctioning EC-cell tumors) are composed of cells with abundant serotonin-storing pleomorphic secretory granules (Fig. 17.23) while less differentiated neoplasms have cells containing few secretory granules with low serotonin content.

17.4.2.7 Tumors Producing Acromegaly, Cushing 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.

17.4.2.8 GRF-, GH, and Ghrelin-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 [308–310]. Very few pancreatic GRF-secreting neoplasms causing acromegaly through GH hypersecretion from hyperplastic pituitary somatotrophs induced by GRF excess have been reported so far [311–316]. Histologically, these tumors show a trabecular, a whorl-like meningotheliomatous or even a paraganglioid "zellballen" structure [193]. They are large tumors, often associated with proven liver or lymph

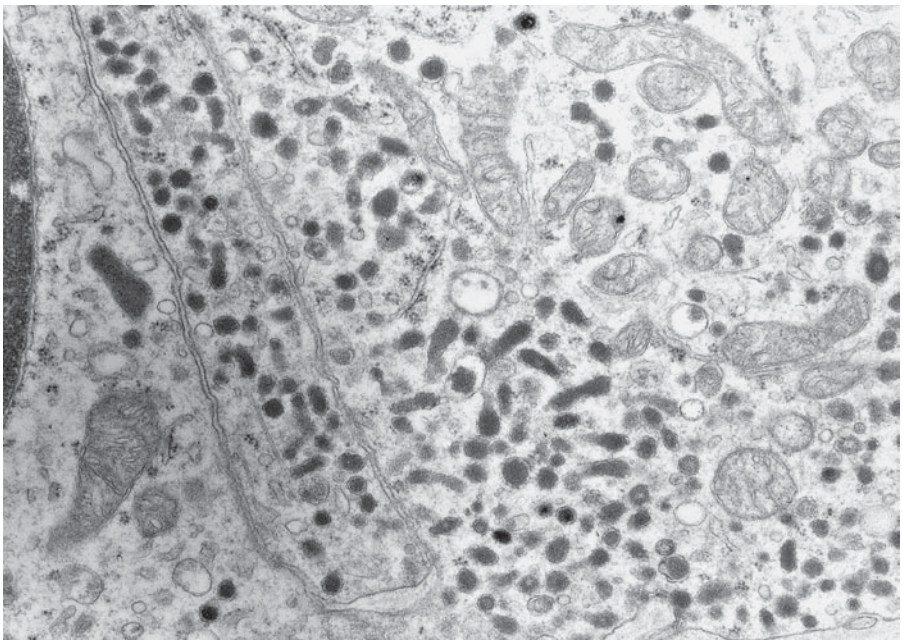

Fig. 17.23 Ultrastructural features of a well differentiated EC-cell tumor. Tumor cells contain abundant pleomorphic secretory granules

node metastases, arising in relatively young subjects (median age of 34 years). Some cases are associated with the MEN1 syndrome [314] or with the Zollinger–Ellison syndrome with concomitant hypersecretion of GRF and gastrin [314]. A single case showing a pituitary metastasis has been reported [311]. In addition to GRF-secreting tumors, a case of GH-producing carcinoma of the pancreas associated with acromegaly has been reported [317]. Acromegalic features and elevated levels of GH and IGF-1 were not present in a case of malignant pancreatic ghrelin-producing tumor with circulating ghrelin level of 12,000 pM [318].

17.4.2.9 ACTH-Secreting Tumors

Pancreatic ACTH-secreting tumors are responsible for about 10% of ectopic Cushing syndrome cases [193, 319–323] and occur most frequently in adults with a prevalence among women, although a few cases have been described in children [322]. In addition, cases associated with the Zollinger–Ellison syndrome have been reported [323]. 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 and bone and display an aggressive biological behavior [319]. 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 (Fig. 17.24). In addition to these well differentiated neoplasms, cases of small cell (poorly differentiated endocrine) carcinomas producing ACTH have also been reported [324].

17.4.2.10 Parathyroid Hormone-Secreting Tumors

A few pancreatic endocrine carcinomas causing hypercalcemia and hyperparathyroid-type syndrome have been observed [325]. In very few cases there was an evident production of parathyroid hormone (PTH) [326–331], while in other cases lacking evidence of PTH secretion an involvement of PTH-related peptide (PTHrP) (parathirin) has been suggested [332]. Interestingly, PTHrP is expressed in the normal islet cells and is commonly detected in PETs [333]. However, elevations of PTHrP alone in the serum are not sufficient to induce hypercalcemia and additional tumor-derived factors are needed to cause hypercalcemia [329].

17.4.3 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 [197, 257]. Among NFETs those causing symptoms due to

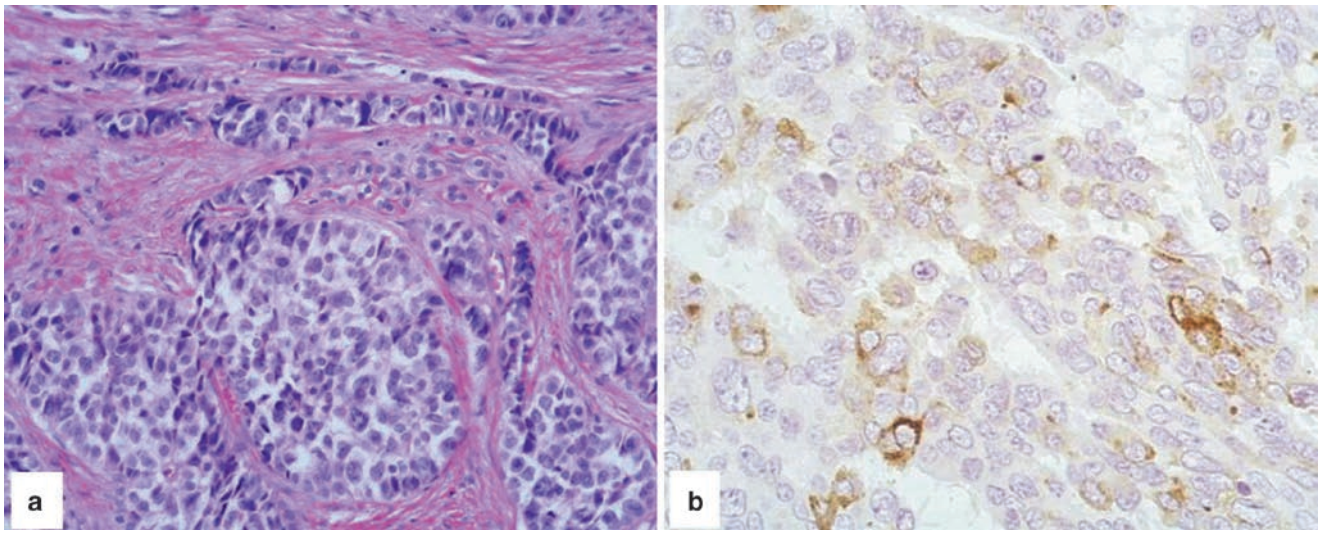

Fig. 17.24 Malignant ACTH-secreting tumor composed of medium-sized cells forming nests and trabeculae separated by fibrous stroma (**a**). Tumor cells are strongly immunoreactive for ACTH (**b**)

the local growth or metastatic spread must be differentiated from incidentally detected clinically silent tumors because the former are mostly malignant (Table 17.7). 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); (6) insufficient clinical investigations [192].

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 about 15–40% of surgically resected PETs [192, 245, 334–336]. Interestingly, NFETs from autopsy series are generally small, benign neoplasms, mainly composed of well granulated α- and PP-cells [337, 338], while the majority of neoplasms from surgical series are malignant and associated with symptoms of an expanding mass [339, 340]. In addition, multiple and small (mainly microadenomas) NFETs are characteristically found in pancreases of MEN-1 patients and are mainly composed of β-, α-, and PP-cells [193, 254]. Moreover, Henopp et al. [341] have recently reported a newly recognized entity named glucagon cell adenomatosis, that resembles multiple and small tumors present in MEN1 patients and, occasionally, in VHL patients [342], but patients with glucagon cell adenomatosis had neither the clinical features nor the genetic findings typical of MEN1 or VHL syndromes. The tumors are mainly composed of glucagon-producing cells and are more frequently clinically nonfunctioning.

Grossly, 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 [343].

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 monomorphic, 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 2 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 [193]. Insulin, when present, is positive in only a few scattered cells while gastrin or VIP immunoreactivities have not been observed [204]. Small incidental nonfunctioning WDETs have a favorable prognosis. On the contrary, locally symptomatic large nonfunctioning WDECs

are usually low-grade malignant with a mean survival of the patients ranging from 23 months to 4.3 years [193, 344].

17.4.3.1 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 [345]. 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 [346].

Calcitonin-cell tumors are generally large (Fig. 17.25) (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 17.12). An association with the MEN-1 syndrome has been described in some cases [346]. In the majority of cases the tumors are malignant, showing metastases at the time of diagnosis. Liver and regional lymph nodes are the more frequent sites of metastases, although brain and bone involvement has also been observed [346].

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. 17.26). Unlike medullary thyroid carcinomas, amyloid deposits have not been observed in pancreatic calcitonin-secreting tumors [345]. 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. 17.26) and, in addition, for other hormones such as PP, αhCG, somatostatin, and neurotensin, in a minority of cells [345, 346]. Follow up data obtained from two series collecting a total of 14 cases are reported in Table 17.13.

17.4.3.2 Pancreatic Polypeptide-Secreting-Cell Tumors

Although scattered PP-immunoreactive cells can be found in several pancreatic endocrine tumors [272, 347] and about 50% of patients with endocrine tumors of the pancreas have an elevated plasma level of PP [348], endocrine tumors composed mainly of PP-cells are rare [193, 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 incidence of this rare tumor type is difficult to assess but it has been evaluated to be about 1–2 per 10^6 population/year [270].

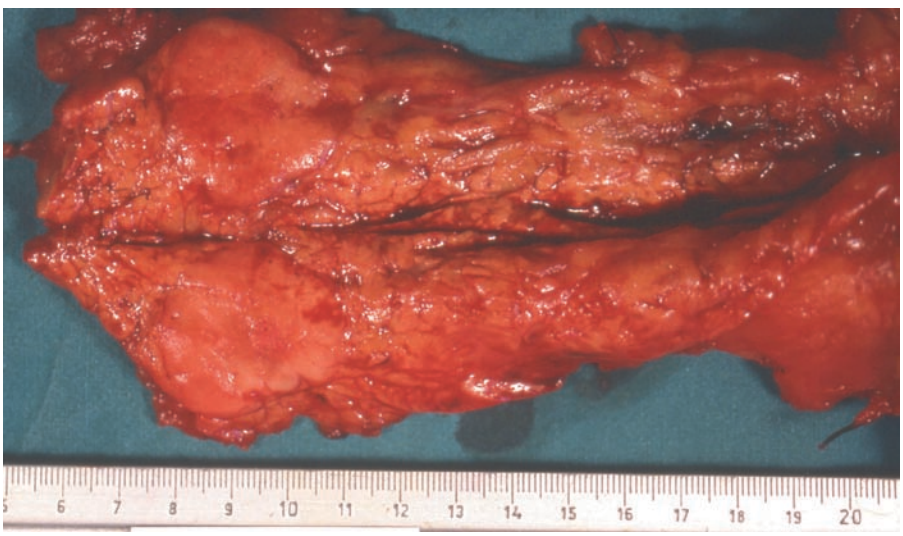

Fig. 17.25 Gross appearance of a malignant calcitonin-secreting tumor of the body of the pancreas measuring about 3 cm. Although the tumor was confined to the pancreas, metastases to regional lymph node were found

Table 17.12 Clinico-pathological features of calcitonin-secreting tumors of the pancreas.

			Site						
SexF/M	AgeMean (range)	*Ø* (cm)Mean (range)	H	T	Met	Vasc inv.	Neur. inv.	Necrosis	References
2/4	52 (30–74)	5.3 (2.5–10)	3	3	5/6				[346]
6/2	46 (30–61)	7.4 (2–20)	4	4	6/8	7/8	5/6	2/8	[345]

Ø diameter; *H* head; *T* tail; *Met* metastases; *Vasc. inv.* vascular invasion; *Neur inv.* perineural invasion

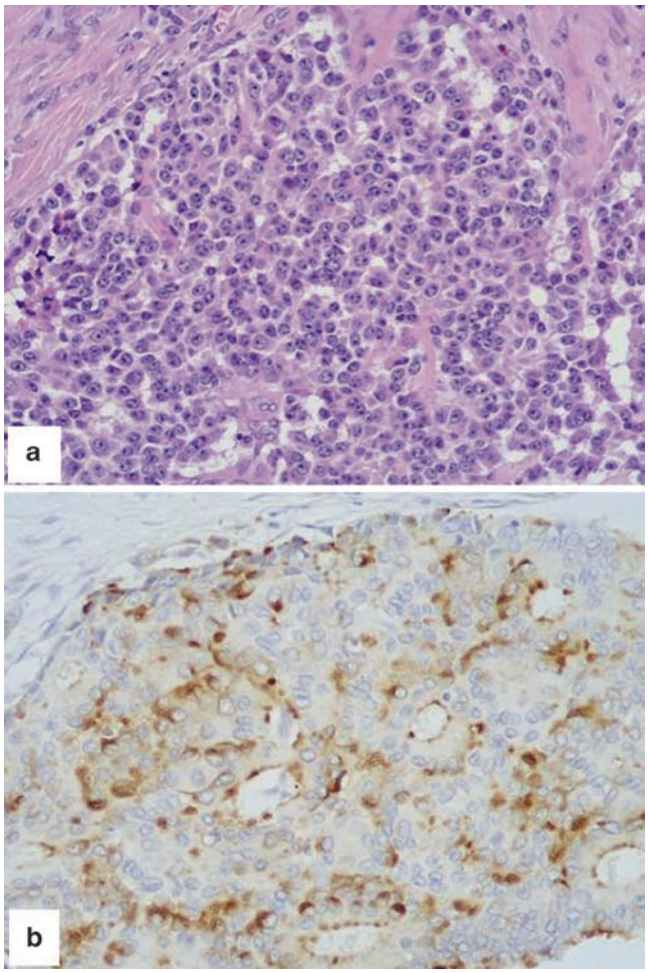

Fig. 17.26 Microscopic features of the tumor shown in Fig. 17.25. Tumor cells are well differentiated mainly growing in a solid fashion (**a**) and are strongly positive for calcitonin (**b**)

Table 17.13 Follow-up data of patients with calcitonin-secreting pancreatic tumors (from references [345, 346]).

6 AFD	18, 18, 24, 36, 77, 112 months, respectively
2 AWD	36, 96 months respectively
3 DOD	2, 10, 24 months, respectively
2 L	
1 DOC	

AFD alive free of disease; *AWD* alive with disease; *DOD* died of disease; *L* lost at follow-up; *DOC* died of other cause

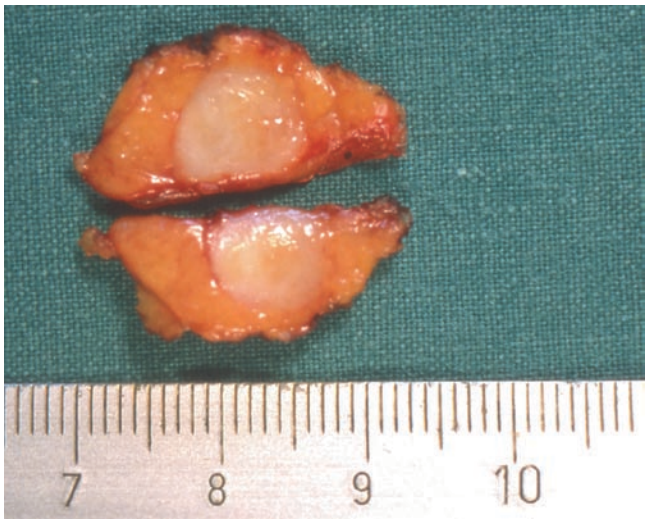

Fig. 17.27 Nonfunctioning PP-cell tumor of the pancreatic head, incidentally found at surgery and enucleated. The tumor is well circumscribed and is about 0.5 cm in size

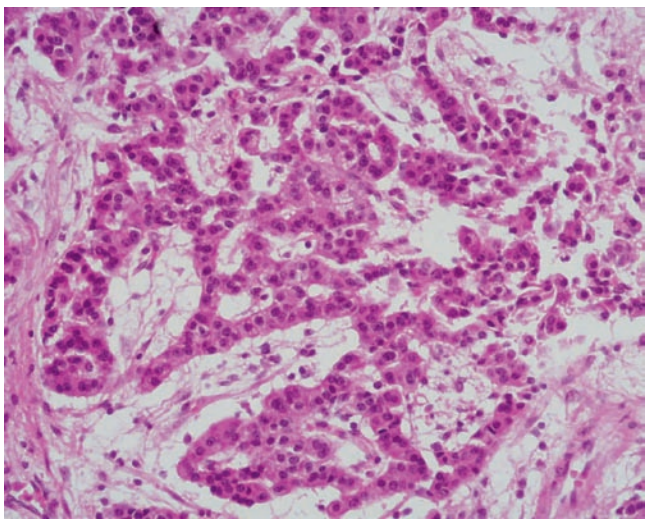

Fig. 17.28 PP-cell tumor formed by trabecular–gyriform structures composed of well differentiated cells without significant nuclear atypia

Among PP-cell tumors, small (less than 2 cm) (Fig. 17.27), 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 [349]. 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. 17.28). 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 in at least 50% of tumor cells.

17.4.4 Poorly Differentiated Endocrine Carcinomas

Poorly differentiated endocrine carcinomas (PDECs) are highly malignant neoplasms composed of small to intermediate (small cell variant of PDEC) and sometimes large cells

(large cell variant of PDEC) showing endocrine features. PDECs make up about 1–5% of all pancreatic malignant tumors [350] and about 2–3% of all pancreatic endocrine tumors of surgical series [193]. They occur in adults, predominantly in men aged between 40 and 75 [351, 352]. 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 extra-abdominal sites is found in practically all cases [193, 351].

Histologically, PDECs show features similar to those of small-intermediate cell carcinoma of the lung. Small to medium-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. 17.29). The mitotic index is high and also the Ki67 proliferative rate as well [204]. PDECs are generally positive for NSE and synaptophysin while granular markers like chromogranins are poorly expressed. Hormone peptide immunohistochemistry is generally negative although ACTH, calcitonin, and somatostatin have been occasionally detected [204, 351, 352]. Unlike well differentiated endocrine tumors, PDECs show diffuse and intense p53 oncoprotein nuclear accumulation [204].

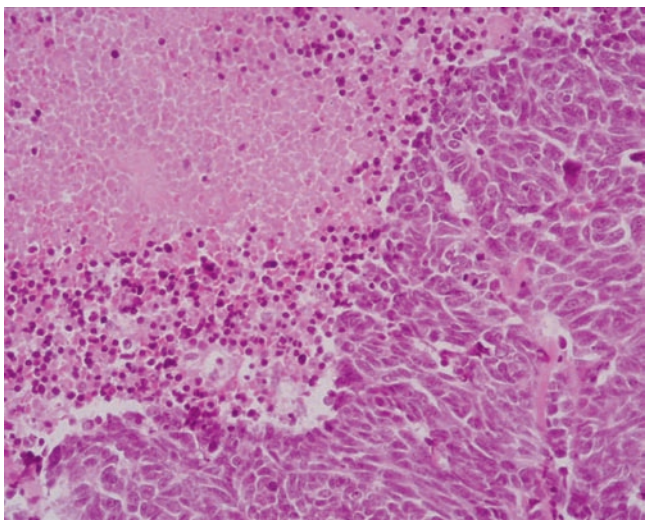

Fig. 17.29 Poorly differentiated endocrine carcinoma of the pancreas composed of cells with severe nuclear atypia forming solid sheets. On the left top an area of necrosis is well evident

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 [353]. Differential diagnosis between PDECs and non Hodgkin lymphomas is based on the positivity in the latter for lymphoid markers such as CD45, CD20 or CD45RO.

17.4.5 Mixed Exocrine–Endocrine Tumors

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 [354]. The exocrine component may be benign (microcystic adenoma) [355, 356] or, more frequently, malignant with the histologic features of either ductal adenocarcinoma or acinar carcinoma [357–359]. The biological behavior of the mixed exocrine–endocrine tumors depends upon 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 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.

17.4.6 Molecular Genetic Alterations in Human Pancreatic Endocrine Tumors

Gastroenteropancreatic endocrine tumors are a poorly understood group of lesions that encompass a broad category of neoplasms derived from endocrine cells of the gastrointestinal mucosa and the pancreas. Among these, PETs are the most studied for molecular genetic alterations. Although most PETs occur sporadically, approximately 5–10% of these neoplasms have a hereditary background and they may be a part of two main hereditary syndromes: Multiple

Endocrine Neoplasia type 1 (MEN1) and von Hippel Lindau disease (VHL). In this section, the current status and recent advances in assessment of the molecular basis of tumorigenesis of PETs will be reviewed.

17.4.6.1 Hereditary Forms of Pancreatic Endocrine Tumors

MEN1 Syndrome

MEN1 (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 MEN1 affected patients [360, 361]. Most of these tumors are small, nonfunctioning, multiple and usually benign (Fig. 17.30). Considering functioning tumors, 54% of affected MEN1 patients develop gastrinomas, 21% insulinomas and less than 5% develop the other functioning PETs [361]. MEN1-associated PETs have an earlier age of 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 MEN1 [361]. The basis of familial MEN1 inheritance is a germline mutation in the MEN1 tumor suppressor gene localized to a small genomic interval at 11q13 by LOH studies and finally identified by positional cloning [362–364]. All MEN1 families reported so far have tight linkage to 11q13 locus and mutation analysis revealed that the MEN1 gene is frequently altered in MEN1 families. Heterozygous germline mutations scattered throughout the MEN1 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) MEN1 related lesions. Of the more than 300 unique mutations reported, over 70% of these are truncating mutations resulting from frame-shift (deletions, insertions, deletion/insertions or splice-site defects) and non-sense mutations [365, 366]. Of the MEN1 missense mutations reported in literature more than 95% occurred at residues highly conserved among human, mouse, zebrafish and Drosophila [366–370], 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 MEN1 in probands and families [371–376]. Most families without demonstrable MEN1 mutations display an atypical clinical pattern, which might suggest genetic heterogeneity of the disease or the occurrence of phenocopies with lesions which are commonly observed in the non-MEN1 individuals, such as primary hyperparathyroidism and prolactinoma [377, 378]. Lastly, when MEN1 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 [378, 379]. 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 MEN1 gene. Menin is a ubiquitously expressed 67-kDa protein found predominantly in the nucleus. 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 as a general transcriptional regulator both targeting a very broad range of promoters in multiple tissues and promoting histone methylation [380, 381]. In both endocrine and non-endocrine tissues, menin was recently proposed to mediate transcriptional activation of genes that inhibit cell growth, including but not limited to, the cyclin-dependent kinase inhibitors p18 and p27. In addition, menin can function as corepressor of tissue-specific genes that promote cell growth, including Hlxb9 [382]. Peripheral blood leucocytes and fibroblasts from MEN1 patients treated with diepoxibutane revealed an increased frequency of spontaneous chromosomal alterations and of mitoses with premature centromere divisions [383–385]. These data derived from in vitro studies are in agreement with recent findings reported by Hessman et al. [386] in a genome-wide LOH screening of 23 MEN1 pancreatic lesions. In this study the authors observed multiple allelic deletions involving chromosome 11, 6, 8, 10, 18, and 21 and a high level of inter and intra tumor heterogeneity suggesting the presence of chromosomal instability. Overall such findings

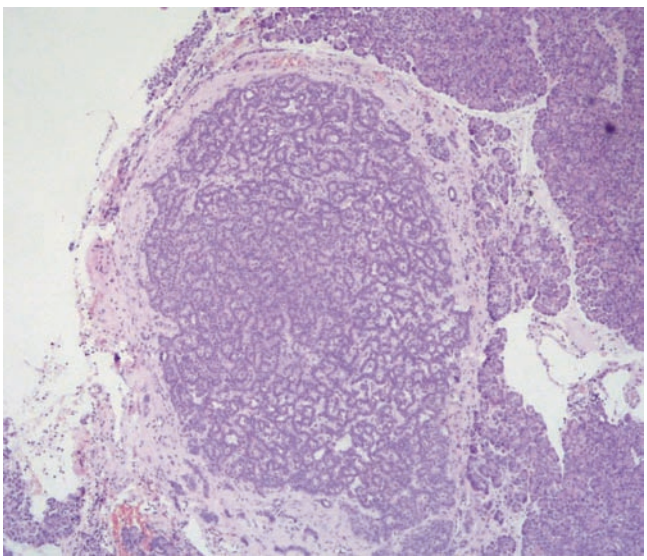

Fig. 17.30 Characteristic pancreatic microadenoma in a patient with MEN1 syndrome

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

Von Hippel–Lindau 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 [387]. VHL-related pancreatic tumors are mostly exocrine microcystic adenomas but 10–15% of patients with VHL could be affected by endocrine tumors [342, 388, 389]. VHL-related PETs have typical morphologic characteristics, consisting of solid, trabecular or glandular structures composed of clear cells with vacuolated lipid-rich cytoplasm in about 60% of cases [390, 391] (Figs. 17.31 and 17.32). 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

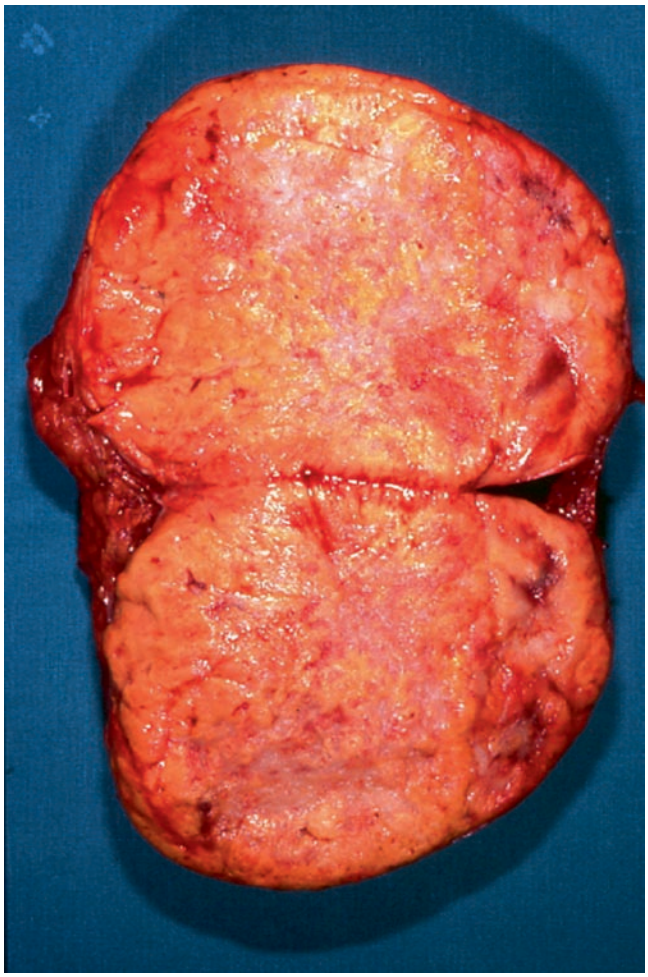

Fig. 17.31 Macroscopic view of a pancreatic endocrine tumor presented in a patient with the von Hippel–Lindau disease. The tumor is well circumscribed, encapsulated and mostly *yellow*

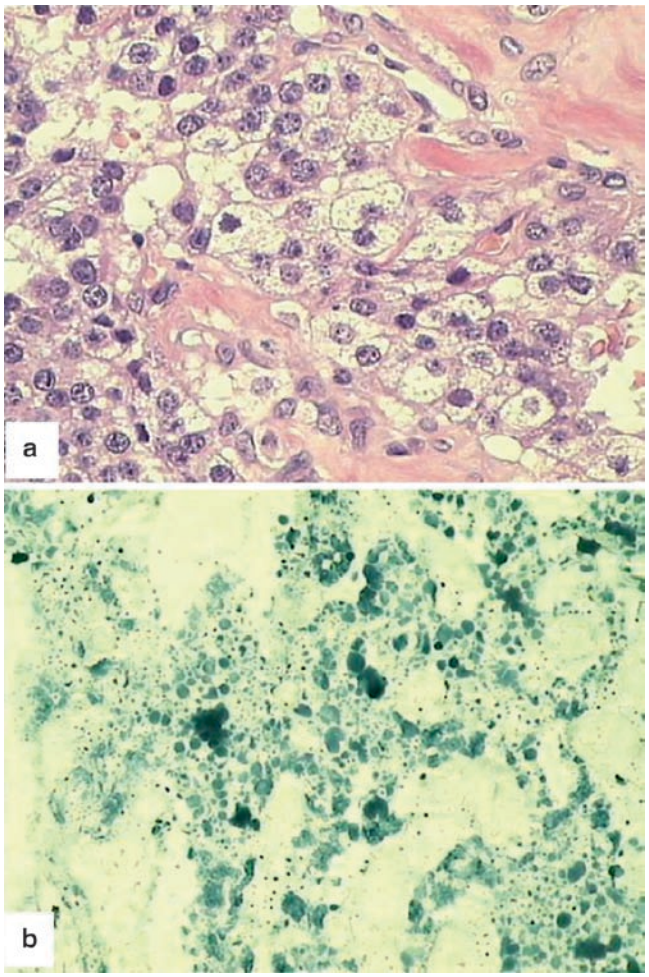

Fig. 17.32 Microscopic appearance of the tumor shown in Fig. 17.31. Tumor cells from the *yellow* areas show a characteristic clear vacuolated cytoplasm (**a**) and were positively stained with Sudan Black stain (**b**)

MEN1-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 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 G0/G1 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 MEN1 and MEN2 [399].

17.4.6.2 Sporadic Pancreatic Endocrine Tumors

More than 90% of PETs are sporadic. Although detailed information is now available on the phenotypic and functional profile of PETs, there is 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 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, 407–409] 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 non-coding 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.

In order 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 while functioning PETs lost only parts of chromosome 3p and rarely the entire chromosome 3p. Since 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 PDECs [204, 413–415].

Controversial data are reported for well-differentiated PETs about mutations of DPC4 gene. Mutations were described in 55% of nonfunctioning tumors as compared to none of the 16 functioning cases [416]. On the contrary, in

recent studies no mutations of 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%).

Although the epigenetic alterations in gastroenteropancreatic endocrine tumors are not well characterized, several studies have recently demonstrated a high frequency of methylation of multiple oncosuppressor genes both in gastrointestinal endocrine neoplasms and in PETs [421, 422]. These findings suggest that gene silencing by DNA methylation may be a crucial event in the endocrine tumorigenesis and also that the methylation status of specific tumor suppressor genes may be a useful marker to predict the response to temozolomide and thalidomide therapy [423, 424].

Other oncosuppressor genes such as RB1, PTEN, BRCA2 [425–427] and common oncogenes including *myc*, *fos*, *c-erbB-2* and *sis* [428] have been examined in PETs but no genetic alterations were observed.

In recent years, in order 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, 429–433]. Genetic alterations seem to accumulate during tumor progression: the total number of genomic changes per tumor appears to be associated with both the tumor size and the stage of the disease [429]. These results point toward a tumor suppressor pathway and chromosomal instability as important mechanisms associated with malignancy in PETs. The alterations described are not randomly distributed on chromosomes but are particularly common in distinct chromosomal regions. Gains are common on 4pq (17% of the tumors) 5q (25%), 7pq (41%), 9q (28%), 12q (23%), 14q (32%), 17pq (31%) and 20q (27%), whereas genomic losses frequently occur on 1p (21%), 3p (19%), 6q (28%), 10pq (14%), 11q (30%), Y (31%) and X (31%). 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. Loss of heterozygosity studies on PETs indicated different chromosomal regions such as 1p, 3p, Xp and 6q, whose deletion is associated with more aggressive behavior [410, 413, 434, 435]. Barghorn [436] 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.

Insulinomas exhibit a lower number of genomic alterations than other PETs [432, 437]. These tumors often have chromosomal aberrations consisting of a gain of 9q32 and loss of 22q13.1, which may occur in the same tumor and appear to be early genetic events in these tumors [438]. Losses of 3p and 6q associated with malignancy are particularly rare in insulinomas [436, 439]. Malignant insulinomas, in contrast, harbor a large number of chromosomal alterations similar to that seen in other types of malignant PETs [433]. In pancreatic gastrinomas, only limited chromosomal imbalances are encountered. Losses at 3p and 18q21 occur in approximately 33 and 22% of cases, respectively [432, 437]. It is interesting to know that 18q losses are also common in gastrointestinal endocrine tumors, indicating that these tumors and pancreatic gastrinomas might be related. Nonfunctioning PETs in general harbor higher numbers of chromosomal gains and losses than functioning tumors. These genetic aberrations occur in chromosomal loci frequently involved in malignant tumors [430]. 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 nonfunctioning 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 the outcome, whereas the ploidy status is an independent factor adding prognostic information to that given by the proliferative index evaluated with Ki-67 immunohistochemistry.

The research on PETs using expression array techniques has only recently started and until now, very few studies have been published to provide specific gene expression profiles with prognostic implications for PETs. The largest published RNA expression array study compared 12 metastasized PETs with 12 nonmetastasized PETs [440]. Malignant PETs compared with non-metastatic PETs, exhibited a downregulation of 51 genes and an up-regulation of 72 genes. These genes

were involved in pathways related to: (1) angiogenesis and remodeling, (2) signal transduction through tyrosine kinases, (3) calcium-dependent cell signaling and (4) response to therapeutic drugs. A second group analyzing the differential gene expression profiles in well-differentiated metastatic PETs and non-metastatic PETs showed an overall pathway activation of the insulin-like growth factor signaling cascade in metastatic PETs compared with nonmetastatic tumors [441, 442]. Capurso et al. [443] described a striking similarity between primary malignant PETs and their metastases concerning their genetic profile, suggesting that the metastatic potential is acquired in an early stage of tumor transformation because cells of the primary tumor already carry the metastatic signatures.

17.4.7 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 [51]. Interestingly, these receptors have a specific distribution in normal islet cell types (Table 17.2) but in PET types they are overexpressed even in cell types that in normal islets lack those particular FGFRs. TGFα has been found in several PETs [444, 445]. However, its specific receptor (EGFR) is expressed at a lower level [446] and frequently only one of the two receptor domains is present, indicating the expression of an incomplete form of the molecule [445]. Hepatocyte growth factor (HGF) has been found to be immunohistochemically expressed in several PETs, including nonfunctioning neoplasms, gastrinomas, VIPomas, and insulinomas [447]. Some of these tumor types also express the HGF receptor, named met [447, 448], suggesting the existence of an autocrine/paracrine mechanism regulating tumor growth. HGF is known to be a stimulator of tumor spread but its expression is not correlated with the metastatic rate in PETs [447]. Insulin-like growth factor, transforming growth factor-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 [80, 449–452], although recent evidences suggests that some of them are associated with more aggressive biology [453].

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Chapter 18
Neuroendocrine Differentiation Patterns in Various Organs (Including Lung, Breast, Skin and Urogenital Tract)

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18.1 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]. These characteristics constitute a vast array of markers, which can now be traced by immunocytochemical procedures, thus acquiring diagnostic interest.

In the gastro-entero-pancreatic (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 lead to the identification of NE cells in other organs such as the 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 the expression of NE markers, possessing relevant diagnostic significance. It was soon realized that NE tumors do not constitute a single, uniform entity, but there is a spectrum in which the degree of NE differentiation matches the clinical behavior, with the well-differentiated tumors being those with a more indolent (benign) course. Tumor entities such as carcinoids, well-differentiated NE tumors and NE carcinomas could thus be established in different organs.

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 site-specific hormones, if any, are being consistently produced by NE cells and tumors of the 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 18.1). 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 the 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 allows to draw information on cell metabolism and on the storage or release of neurosecretory granules as well. Chromogranin A (CgA), the most widely and intensely expressed member of the family, is stored in high 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 lung, breast, skin, prostate and urothelium.

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Table 18.1 Neuroendocrine cell markers.

Fluorogenic amine content
Amine precursor (5-hydroxytryptophan and DOPA) uptake
Aromatic amino acid decarboxylase
Non specific esterase or cholinesterase
Alpha 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 vescicle proteins
Lymphoreticular antigens
Tetanus toxin-binding sites

18.2 Lung

18.2.1 NE Cells in Normal Lung and in Non-neoplastic Conditions

NE cells (also called Kulchitsky or K cells) of the lung which are part of the diffuse NE system, are of both opened and closed types, and occur as solitary cells or in small aggregates, and are 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 week 16 to 30 of gestation and decreases at about 6 months 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 ACTH, 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 which include (a) increased number of scattered NE cells, (b) linear proliferations along the bronchial mucosa, and (c) nodular hyperplasia consisting of increased number of NE bodies. While these proliferations can be related mainly to chronic bronchial inflammation [7], Langerhan 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 less than 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 relation between high-grade NE tumors, as small cell and large cell carcinomas, and hyperplastic NE proliferations seems less convincing.

18.2.2 Pathological Classification of Pure NE Tumors

Neuroendocrine tumors of the lung share most morphological and clinical features observed in NE tumors of other organs, and, rather than being a uniform entity, build up a spectrum of lesions associated with specific pathological features [11]. Their classification combines architectural patterns (i.e., organoid vs. diffuse growth) with other pathological parameters, such as mitotic index and the presence of necrosis, for the purpose of recognizing four different entities with significant differences in terms of clinical behavior and survival [12–15]. The spectrum of pure NE tumors, including typical and atypical carcinoids, large cell neuroendocrine carcinoma, small cell carcinoma, combined carcinomas and non-NE carcinomas with focal NE differentiation, as well as their main pathological and clinical features are extensively illustrated in Chap. 19. In the next paragraph we will briefly focus on the molecular features of these tumors.

18.2.3 Molecular Profile

The molecular profile of NE lung cancer has been extensively investigated at genome, gene transcript, cell product, receptor and regulatory peptide expression levels, with the aim at identifying features that might be complementary or alternative to morphology to better define the different histological types and address a more appropriate therapeutic strategy.

Wide-genome screening, by means of comparative genomic hybridization, or allelotyping identified chromosomal abnormalities specific for different NE tumor histotypes. The most common genetic feature in both typical and atypical

carcinoids is the allelic deletion of the long arm of chromosome 11, which is more frequently lost (nearly one third of sporadic carcinoids) at 11q13, the MEN1 gene locus, that is inactivated by either loss of heterozygosity (LOH) or microsatellite instability (MSI), with the consequent absence of the gene product menin [16]. However, the development of lung carcinoids in MEN1 syndrome is a rare occurrence. MEN1 gene alterations are also virtually absent in poorly differentiated large cell and small cell NE carcinomas, and MEN1 kindreds do not present these types of tumors as inherited carcinomas. Interestingly, familial lung carcinoid syndromes different from MEN1 have been described, and still need to be better clarified [17].

With regard to small cell lung carcinoma, the vast majority shows deletions of the short arm of chromosome 3, being the putative tumor suppressor genes still to be completely identified. The von Hippel Lindau (3p25) and FHIT (3p14.2) genes seem the most likely candidates, and LOH of both has been detected in a high percentage of cases. However, although the frequency of LOH at 3p was higher in the group of high-grade NE carcinomas, large and small cell types had different 3p LOH patterns and a significantly different genetic background was observed in these two forms [18].

Concerning key pathways which regulate the cell cycle, *P53* gene point mutations are frequently present in large cell NE carcinomas and small cell carcinomas, while absent in all typical and most atypical carcinoids [19]. In this context, immunohistochemical detection of p53 protein has been proposed in the differential diagnosis of NE lung tumors. Mutations in *RB1* and *PTEN* genes have also been detected in most small cell lung carcinomas [20, 21]. At the protein level, heterogeneous expression patterns of proteins acting in the cell cycle control have been described, probably reflecting variable genetic diversity among individual tumors, with a generally increased expression of Rb/p16/cyclin D1 and E2F-1 pathway molecules in high-grade NE tumors [22, 23].

High-grade NE lung tumors, as compared to carcinoids, generally over-express several tyrosine kinases, such as VEGF receptors and c-Kit, but activating mutations of the corresponding genes are extremely rare. In contrast, a high prevalence of activating mutations of the neurotrophic tyrosine receptor kinase type 2 and 3 genes, exclusively restricted to the large cell NE carcinoma subtype, has been recently demonstrated [24].

At the level of gene expression profiling, some reports found that carcinoids (typical and atypical) cluster with gliomas, while small cell carcinoma cluster with bronchial epithelium, suggesting a different histogenesis of these two tumors subtypes [25]. Interestingly, large cell NE and small cell carcinomas are indistinguishable by gene expression profiling, that conversely identifies two separate groups, irrespective of the large or small cell carcinoma histotype, with relevant differences in term of prognosis [26].

The development of novel therapeutic strategies in NE tumors, targeted to peptide receptors, signaling molecules or catalytic enzymes, led to several reports aimed at the definition of specific phenotypes predictive of therapeutic response. Among peptide receptors, somatostatin receptors have been demonstrated in NE lung tumors by means of alternative techniques [27], although the use of somatostatin analogs in the diagnosis/medical therapy of NE lung tumors is less standardized as compared to the corresponding neoplasms of the gastrointestinal tract. Among potential predictive markers of response to chemotherapy, mRNA expression of thymidylate synthetase, a selective target of antifolate drugs, has recently been demonstrated to correlate with clinical response in a group of patients treated with 5-fluorouracil, including lung NE tumors [28]. An alternative novel target is represented by mTOR (the mammalian target of rapamycin), a pivotal molecule involved in the regulation of cell growth and proliferation; its activity is selectively blocked by a family of molecules, including rapamycin, everolimus and temsirolimus, that in preliminary experimental models of small cell carcinoma cell lines proved to overcome chemoresistance and promote apoptosis [29].

18.3 Breast

18.3.1 NE Cells in Normal Breast

The presence of argyrophilic and Chromogranin A-reactive cells, located between the basal myoepithelial and the luminal epithelial cells, have been occasionally demonstrated in histologically normal breast tissue surrounding infiltrating endocrine breast carcinomas [30] (Fig. 18.1). However, nothing is known on specific hormonal peptides produced by breast tissue.

18.3.2 Neuroendocrine Breast Carcinoma

18.3.2.1 Definition

Reports on breast tumors showing features similar to carcinoids date back to 1963 [31]. Some years ago, we defined as "endocrine differentiated breast carcinomas" a subset of tumors whose morphology, histochemical and immunocytochemical features overlap those of neuroendocrine carcinomas of the gastrointestinal tract, lung, prostate and other organs [32]. Following a quantitative approach, NE breast cancers are tumors that express NE markers in more than 50% of their cell population [33]. Only 2 to 5% of breast

carcinomas fall within this specific definition, however the percentage increases in the 6th or 7th decades of life [34].

18.3.2.2 Cytology

Cytological features of NE carcinomas are very peculiar and useful for diagnosis [35] (Fig. 18.1). The NE cells are plasmacytoid, spindle or with signet ring appearance and show intracytoplasmic granules particularly evident with Giemsa and Diff-Quick stains. Dark hypercromatic nuclei, "crush artifacts" and nuclear streaming are conversely the hallmarks of small cell carcinoma on cytological samples.

18.3.2.3 Histotypes

Neuroendocrine ductal carcinoma in situ (NE-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. As compared to conventional DCIS, NE-DCIS usually shows lower nuclear grade and proliferation index, and higher expression of estrogen and progesterone receptors with a negative HER2 score in the vast majority of cases [36].

Solid-cohesive carcinoma or low-grade insular carcinoma is the morphological variety more similar to carcinoid tumors (Fig. 18.1). In fact, this infiltrating carcinoma is formed by highly cellular nests, or trabeculae of NE cells, with peripheral cell palisading, reminiscent of carcinoid tumors. Rarely, the cells are organized in glandular or rosette-like structures. Neuroendocrine 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 alveolar-like structures, separated by scant dense stroma, that produce an infiltrative growth pattern similar to the alveolar variant of lobular carcinoma.

Mucinous differentiation is observed in 26% of NE carcinomas of the breast [34]. In NE 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 [37] adopted the descriptive term "cellular mucinous carcinoma" to differentiate the NE variant of mucinous carcinoma from the non-NE one. In the so-called "solid papillary carcinoma," solid round nests of cells with papillary features intermingle with small lakes of extracellular mucin [38].

Following the classical criteria of grading, all the above described varieties of NE carcinomas are well or moderately differentiated tumors expressing estrogen and progesterone receptors. About 50% of such tumors express Chromogranin A (Fig. 18.2) and B and synaptophysin [34].

Poorly differentiated NE carcinoma represent 15% of NE breast carcinomas. The diagnosis of poorly differentiated NE carcinoma is suggested by the extremely high number of mitoses ranging from 18 to 65 per 10 hpf, in a tumor which maintains the NE architecture and expresses chromogranin A [34].

Finally, small cell/oat cell carcinomas of the breast have 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. NSE is expressed in the vast majority of small cell carcinomas of the breast, whereas chromogranin A and synaptophysin are expressed in about 50% of such cases.

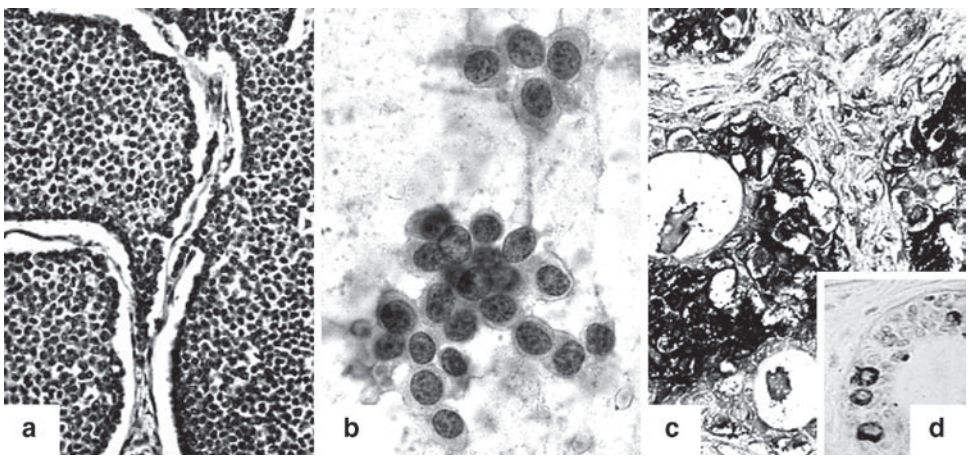

Fig. 18.1 Endocrine breast carcinoma. Monomorphic cells grow in solid chords (**a**) and present a cytological plasmocytoid appearance (**b**) with round nuclei showing mild atypia. Neoplastic cells are strongly positive for chromogranin A, as compared to negative ductal structures (**c**). Peritumoral ducts contain single normal endocrine cells, as detected by chromogranin A imunohistochemistry (**d**) [(**a**) H&E, ×200; (**b**) H&E, ×800; (**c**) immunoperoxidase, ×400; (**d**) immunoperoxidase, ×200]

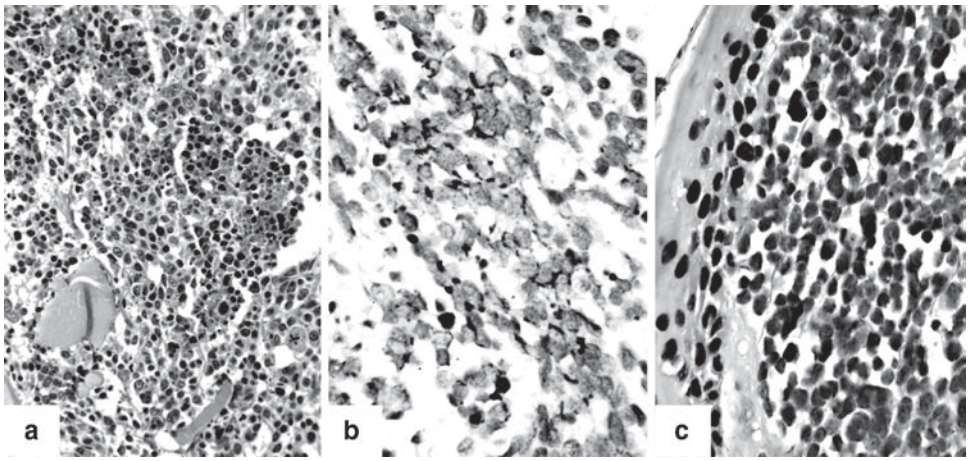

Fig. 18.2 Merkel cell carcinoma. Small-sized neoplastic cells with marked atypia, focal necrosis and high mitotic activity, grow in a diffuse fashion (**a**) and express epithelial (pan-cytokeratins in peculiar dot-like appearance) markers (**b**); nearly half of the cases express the basal cell differentiation marker p63 (**c**) which is correlated to adverse outcome [(**a**) H&E, ×200; (**b**) immunoperoxidase, ×200; (**c**) immunoperoxidase, ×400]

In addition, 20% of small cell mammary carcinomas express TTF-1 [39]. Primary mammary small cell carcinomas are cytokeratin 7-positive and cytokeratin 20-negative, whereas, for example, lung small cell carcinomas are usually negative for both. In addition, estrogen receptors are expressed in more than 50% of small cell carcinomas [39].

However, immunodetection of pan-endocrine markers may fail to recognize NE tumors, which produce but do not retain the specific antigen in the cells. When the morphological suspect of the NE nature of a tumor does not correlate with the immunophenotype, the lack of high molecular weight cytokeratins, which selectively label non-NE carcinomas, may support the NE tumor diagnosis and induce different types of analysis [39].

18.3.3 Divergent Differentiation

A relatively common phenomenon in NE 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 NE breast carcinomas, and we have demonstrated that apocrine differentiation is present in about 50% of well and moderately differentiated NE breast carcinomas as well [34], while poorly differentiated carcinomas do not show this multi-differentiation capacity and express the endocrine markers, only.

The immunocytochemical and in situ-hybridization co-expression 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 NE and apocrine lineage. Interestingly, expression of apocrine differentiation markers in NE breast carcinomas is typical of aged women and androgen receptors are expressed by 80% of cells of neuroendocrine-apocrine differentiated tumors [34]. 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.

18.3.4 Clinico-Prognostic Parameters

A major matter of concern stems from the impact on the patient's clinical history and prognosis, when a diagnosis of NE carcinomas of the breast is made. While the histological patterns above described are useful to correctly diagnose a NE carcinoma, production and secretion of specific markers and the histological grade of differentiation are the two factors with major clinical and prognostic impact. Evaluation of chromogranin A or NSE serum levels may be taken into account in the patients' follow up.

Regarding the clinical evolution of NE breast carcinomas, we demonstrated that similar to conventional breast carcinomas, the histological grade is one of the most important parameters to predict clinical evolution [40]. Poorly differentiated NE 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, all patients being alive at long-term follow-up. Mucin production is correlated to 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 [34].

18.3.5 Non-NE Carcinomas with Focal NE Differentiation

Carcinomas not otherwise specified which show immunocytochemical expressions of NE markers by scattered cells [33] are excluded from the subset of NE breast carcinomas. Focal expression of NE markers in breast carcinomas is in line with similar observations in other exocrine tumors of endodermal (lung, gastro-intestinal tract) and ectodermal (skin) origin, and does not carry any clinical or prognostic significance [41].

18.4 Skin

18.4.1 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 [42]. It was found in many species, including mammals and man among them. The oral mucosa and lips also contain scattered Merkel cells [43]. 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. Merkel cell have been found to produce several hormonal peptides, including VIP and bombesin [43]. The immunophenotypic profile of Merkel cells includes expression of pan-endocrine markers, namely chromogranin A, 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 at electron microscopy examination. Hyperplasia of skin NE cells 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 [43].

18.4.2 Merkel Cell Carcinoma

Merkel cell carcinoma is a very rare malignant skin tumor, described 30 years ago [44] 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.

18.4.2.1 Synonims

This tumor is reported under several labelings, 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, Merkel cell carcinoma.

18.4.2.2 Clinical Data

The exact incidence is not known. It is a very rare disease with an incidence progressively increasing from 0.15 to 0.44 per 100.000 annually, from 1986 to 2001 [45]. It affects the Caucasian elderly population (over 65 years old) with no sex preference. The classical location is the head&neck area (up to 75% of cases in some series), the other cases being almost equally distributed in the limbs and trunk [46]. The majority of tumors tend to be asymptomatic despite their rapid growth and frequent nodal involvement at the time of diagnosis [45].

18.4.2.3 Gross Features

Merkel cell carcinoma appears as a reddish to pink small dermal nodule or as a plaque of approximately 2 cm which may ulcerate the overlying skin or discolorate it. Multiple small satellite nodules around the primary tumors have also been described. About 14% of cases present as primary lymph node disease without apparent cutaneous involvement [45].

18.4.2.4 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-sized or small cells (Fig. 18.2a), 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 non-cohesive 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 ovoidal with centrally located nuclei 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) has been only occasionally observed in Merkel cell carcinoma and, if extensively present, better 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 which are to be distinguished from small tumor cells, especially in the evaluation of surgical margins of frozen sections. Merkel cell carcinoma may be associated with other lesions. The most commonly encountered is in situ or invasive squamous cell carcinoma, either in the primary Merkel cell carcinoma or in its lymph node metastases. More rarely described are glandular differentiation or a lymphoepithelioma-like carcinoma component in a Merkel cell carcinoma 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 [47].

18.4.2.5 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 dot-like paranuclear reaction (Fig. 18.2b). High molecular weight cytokeratins (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 non-endocrine skin carcinoma. Pan-endocrine markers are variably (20–100%) expressed in Merkel cell carcinoma, including NSE, neurofilament proteins, synaptophysin and chromogranin A. Finally, reactivity for several different hormones has been reported, including ACTH, calcitonin, somatostatin, VIP, bombesin and others.

18.4.2.6 Behavior

Merkel cell carcinoma is a highly invasive tumor, which frequently infiltrates the 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-year 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%) [48]. Adverse prognostic parameters include deep tumor infiltration, nodular growth pattern, presence of lymphovascular invasion and high tumor stage [46]. Recently, immunohistochemical markers such as survivin have been correlated to prognosis in Merkel cell carcinoma [49]. Moreover, our group recently demonstrated that the expression of the basal cell differentiation marker p63 is strongly correlated to proliferation and adverse outcome in Merkel cell carcinoma (Fig. 18.2c) [50].

The treatment of choice is surgical, with wide excision of at least 3-cm margins and dissection of palpable regional nodes. Radiation therapy may follow. Advanced cases do not gain any benefit from radio-or chemotherapy. Hormonal treatment with somatostatin analogs has also been reported in a few cases [51], based on the expression of somatostatin receptors in Merkel cell carcinoma [52, 53].

18.4.2.7 Differential Diagnosis

Merkel cell carcinoma may share several morphological similarities with small cell carcinoma of the lung and other locations. Dot-like 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 [54]. The combined evaluation of a panel of markers, which include cytokeratin 20, neurofilament proteins, TTF-1 and human ASH-1 [55, 56], will address a correct diagnosis. The diffuse non-cohesive variants of Merkel cell carcinoma has to be distinguished from non-Hodgkin 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 18.2).

18.4.2.8 Molecular Profile

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 [57]. A more detailed chromosomal analysis is mapped at 1p34, a hotspot region of amplification corresponding to the L-Myc locus [58]. Concerning oncogenetic protein expression in Merkel cell carcinoma, VEGF isoforms and VEGF receptors, c-kit and PDGF receptor alpha (but in the absence of activating gene mutations) together with their corresponding ligands, and the apoptotic/cell cycle-related molecules Mcl-1 and Bmi-1 showed significant expression rates [59, 60], without any significant correlation with the clinical outcome.

Table 18.2 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	+ (dot-like)	+	+	–
HMW Cytokeratin	–	+	–	–
Endocrine markers	+	–	+	–
Lymphoid markers	–	–	–	+

ca carcinoma; *LMW* low molecular weight; *HMW* high molecular weight

Very recently, the clonal DNA integration of a previously undescribed virus, then named Merkel cell polyomavirus, has been demonstrated in a high proportion in Merkel cell carcinomas [61, 62], and strongly supports its possible role as an etiologic agent in Merkel cell carcinoma carcinogenesis.

18.4.3 Primary Carcinoids of the Skin

They are exceedingly rare. Very few cases are on record in the English literature to date [63]. 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 as classically observed in the appendix. The other were insular or trabecular well-differentiated endocrine tumors, rarely with atypical features such as moderate mitotic index.

The phenotype was that of typical carcinoids with panendocrine marker expression, argyrophilia and 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.

The morphological appearance was overlapping with that of the more common metastases of carcinoid tumors to the skin and the final diagnosis is largely based on the exclusion of primary endocrine tumors in other locations.

18.4.4 NE Differentiation in Non-endocrine Carcinomas of the Skin

18.4.4.1 Basal Cell Carcinoma

The presence of endocrine cells in basal cell carcinoma was originally described in 1979 by Eusebi and co-workers [64], using argyrophilic methods and electron microscopic demonstration of dense-core neurosecretory granules. Subsequent reports demonstrated several NE products (both hormones and pan-endocrine markers) in basal cell carcinomas by means of immunohistochemistry and/or RT-PCR [65, 66]. 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 a NE phenotype in an otherwise classical basal cell carcinoma rather than to the entrappement of normal Merkel cells of the skin, since no parallel cytokeratin 20 immunoreactivity (marker of Merkel cell carcinoma) was ever found in these tumors [47].

18.4.4.2 Adnexal Carcinoma

Both apocrine and mucinous eccrine carcinomas have been reported [47]. In the former, classical apocrine eosinophilic cells are admixed with a minor cell population showing argyrophilia and chromogranin A expression. In the latter, a classical mucinous carcinoma, as commonly found in breast and

colorectal locations, displays single chromogranin A-reactive cells in the tumor cell nests and sparse in the mucinous lakes.

18.4.4.3 Follicular Differentiated Tumors

Some reports [67] 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 to be related to a hyperplastic condition rather than to a neoplastic divergent cell population.

18.5 Prostate and Urothelium

18.5.1 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 nerve-like varicosities extending between adjacent epithelial cells and occasionally abutting on other NE cells (Fig. 18.3a). Ultrastructural analysis shows a wide variety of neurosecretory-type granules containing different secretory products, including serotonin and various peptides, such as the chromogranin family, the calcitonin family, a thyroid-stimulating hormone-like peptide, bombesin/gastrin releasing peptide, somatostatin and parathyroid hormone-related protein [68–72]. NE cells lack expression of the androgen receptor [73].

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. Nerve processes seen in association with prostatic NE cells suggest 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 [74].

In the urinary bladder, NE cells were first described by Feyrter [75]. Fetissof [76] established that the NE 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. 18.3b). Little is known about their endocrine functions, but immunohistochemical studies demonstrated that they produce serotonin but lack peptide hormones such as ACTH, gastrin, glucagon or somatostatin.

18.5.2 Well-Differentiated NE Tumors and Carcinomas

Well-differentiated NE tumors (carcinoid) and carcinomas in the urogenital tract are extremely rare, occurring isolated or combined with classic adenocarcinoma [77]. In the prostate, generally PSA levels are not elevated. Rarely hormonal symptoms are present [78]. Histologically, NE tumors and carcinomas of the urogenital tract show carcinoid-like appearance characterized by solid, acinar and cribriform structures formed by cuboidal and columnar cells. NE carcinomas, in contrast to NE tumors, show either necrosis or vascular and/or perineural invasion or metastases to lymph nodes and distant organs. Proliferation index evaluated with MIB-1, is low and can help to differentiate these tumors from small foci of undifferentiated, malignant solid or trabecular urothelail or prostate carcinomas (Table 18.3).

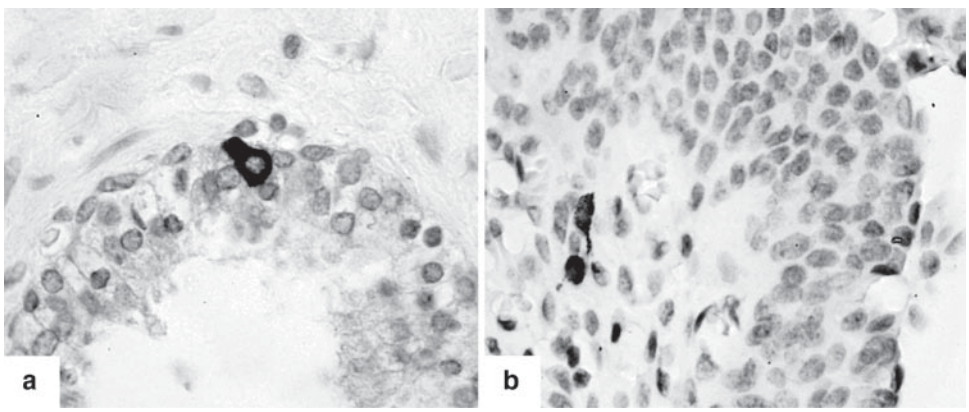

Fig. 18.3 Normal neuroendocrine cells in the prostate (**a**) and urinary bladder (**b**), both of the closed type, as detected by chromogranin A immnuhistochemistry [(**a**, **b**) immunoperoxidase, ×400]

Table 18.3 Immunohistochemical markers in the differential diagnosis of NE tumors of the prostate and urinary bladder.

	ChA, SNP	HMW-CK	PSA	AR	CLA	Ki-67
NET/Low-grade NEC bladder/prostate	+	–	–	–	–	Low
High-grade NEC bladder/prostate	+[a]	–	–	–	–	High
Poorly differentiated ca. Prostate	–	–	+	+	–	Intermediate
Poorly differentiated ca. bladder	–	+	–	–	–	Intermediate
Paraganglioma	+	–	–	–	–	Low
Lymphoma	–	–	–	–	+	Variable

ChA 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 ChA

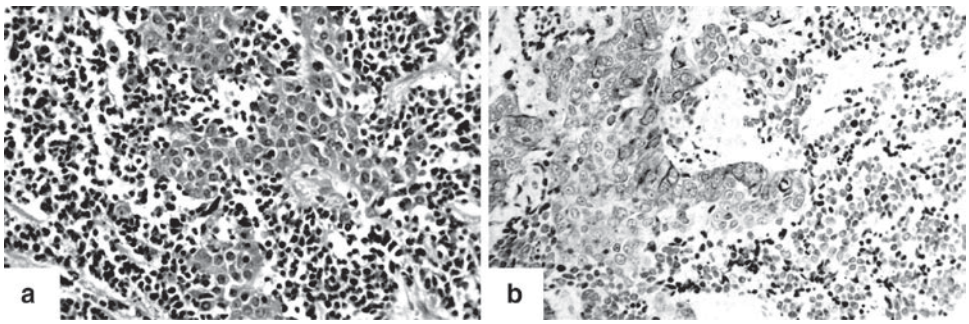

Fig. 18.4 Poorly differentiated neuroendocrine carcinoma of the bladder. A mixed small and large cell neoplastic population is shown (**a**), lacking expression of high molecular weight (34βE12) cytokeratins (**b**) [(**a**) H&E, ×200; (**b**) immunoperoxidase, ×200]

18.5.3 Poorly Differentiated (Small Cell and Large Cell) NE Carcinomas

Most NE carcinomas of the prostate and almost all NE carcinomas of the urinary bladder (first described by Cramer in 1981) [79] belong to the poorly differentiated or high-grade category [80–82]. The frequency of high-grade NE carcinomas, when compared with all carcinomas of both organs, is very low, ranging from 0,2 to 1,0%. The tumor usually affects patients older than 50 years.

From a morphological and clinical point of view, they do not differ from small cell carcinomas or large cell NE carcinomas from other sites (Fig. 18.4). In prostatic tumors, as for well-differentiated tumors and carcinomas, PSA levels generally are not elevated. Paraneoplastic syndromes due to ectopic hormone production may be observed, including Cushing's syndrome, myasthenic (Eaton-Lambert) syndrome, malignant hypercalcemia and inappropriate antidiuretic hormone secretion syndrome.

18.5.4 Mixed High-Grade NE and Non-NE Carcinomas

Prostatic small cell carcinomas can be associated with classic adenocarcinoma [78]. Moreover, an association with sarcomatoid and squamous carcinoma components can occur. In the urinary bladder, up to 50% of small cell carcinomas are associated with areas of transitional cell carcinoma [83].

18.5.5 Non-NE Carcinomas with Focal NE Differentiation

Among urogenital tumors, the occurrence of NE differentiation in non-NE tumors have been described almost exclusively in the prostate. Almost all adenocarcinomas of the prostate contain isolated NE cells. Focal NE differentiation

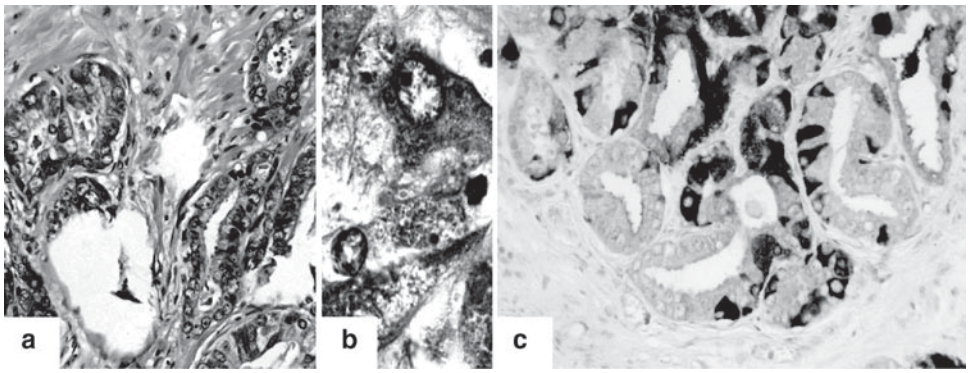

Fig.18.5 Adenocarcinoma of the prostate having neuroendocrine differentiation features. Neoplastic glands contain large cells with abundant granular cytoplasm, having a characteristic Paneth-like appearance (**a**, **b**). These same cells strongly express chromogranin A (**c**) [(**a**) H&E, ×200; (**b**) H&E, ×1,000; (**c**) immunoperoxidase, ×200]

is seen in virtually all prostate carcinomas, but is very uncommon in bladder cancer [84].

NE differentiation cannot usually be appreciated without special immunostainings, as peculiar morphologic changes are almost absent, exept in those cases showing a Paneth cell-like change ("red cells" within the glands, containing large eosinophylic secretory granules) [85] (Fig. 18.5). 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 vescicular with prominent nucleoli. Lysozyme immunoreactivity is invariably negative.

The biological significance of NE differentiation in prostate cancer is incompletely elucidated. It has been suggested that the coexistence of NE phenotype in predominantly exocrine tumors correlates to overall prognosis [86] and seems of special importance in facilitating prostate cancer progression after androgen-deprivation therapy. Several mechanisms have been identified in this respect: NE cells are androgen receptor negative, therefore they survive to androgen deprivation; as an alternative, NE cells produce peptides, hormones and growth factors that could stimulate the proliferation of exocrine prostate cancer cells and increase their aggressiveness through apoptosis inhibition and neoangiogenesis stimulation [87–89]. Two recent studies in patients with hormone refractory disease have demonstrated an independent poor prognostic role of elevated circulating CgA levels [90, 91]. In patients with hormone naive disease, however, despite several evidences showing a significant relationship between the amount of NE cells and disease stage and grade [92, 93], the relationship of NE differentiation with disease-free survival and overall survival is still controversial.

Androgen suppression by either orchiectomy or administration of luteinizing hormone-releasing hormone analogs (LHRH-A) is the cornerstone of advanced prostate cancer management [94], and this treatment modality is increasingly prescribed in the early phases of the natural history of the disease. NE differentiation regulatory mechanisms might play a role in the transition from an androgen-dependent to an androgen resistant phenotype in prostate cancer. In fact, in vivo studies, both in animals [95] and humans [96], have shown that the NE prostate cancer compartment increases after androgen deprivation, and the up-modulation by androgen withdrawal of specific transcription factors involved in neuroendocrine cell development and differentiation has been recently proposed as a pivotal mechanisms [97].

18.5.6 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 and are characterized by typical "zellballen" growth pattern, the cells being positive for conventional NE markers.

In the urinary bladder, these tumors may look like polyps which can be resected by simple excision. Paragangliomas are sometimes incidental findings after radical cystectomy. Presenting symptoms are haematuria and attacks of hypertension during micturition. Multifocality is present in 20% of cases. They can metastasise 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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Chapter 19
Endocrine Lung

William D. Travis

19.1 Introduction and Classification

Neuroendocrine lesions of the lung include neuroendocrine cell hyperplasia and tumorlets, and a spectrum of pulmonary neuroendocrine tumors that includes the low-grade typical carcinoid (TC), intermediate-grade atypical carcinoid (AC), and the high-grade tumors of large cell neuroendocrine carcinoma (LCNEC) and small cell carcinoma (SCLC) (Table 19.1). Diffuse idiopathic neuroendocrine cell hyperplasia is a very rare condition that represents a pre-invasive lesion for carcinoid tumors. Despite the common neuroendocrine properties of TC, AC, LCNEC, and SCLC, there are major clinical, epidemiologic, and genetic differences between carcinoid tumors and the high-grade SCLC and LCNEC [1–5]. Both TC and AC can occur in patients with Multiple Endocrine Neoplasia (MEN) type I but LCNEC and SCLC do not [6]. In contrast to LCNEC or SCLC, both TC and AC can have associated neuroendocrine cell hyperplasia with or without tumorlets. Also, both LCNEC and SCLC can demonstrate histologic heterogeneity with other major histologic types of lung carcinoma, such as adenocarcinoma or squamous cell carcinoma, but is not characteristic of TC or AC [6–8]. The diagnosis of SCLC, TC, and AC can be made by light microscopy without the need for special tests in most cases, but for LCNEC it is required to demonstrate neuroendocrine differentiation by immunohistochemistry or electron microscopy. In some cases, immunostains are helpful in sorting the differential diagnosis, especially in small biopsies. Finally, genetic changes are very frequent in SCLC and LCNEC, but usually uncommon in TC and intermediate in AC [9–13]. Neuroendocrine tumors of the lung comprise approximately 20–25% of all invasive lung malignancies. Small cell carcinoma is the most common of the neuroendocrine lung tumors accounting for 15–20% [14], followed by LCNEC which is about 3% in surgical series and carcinoid accounts for 1–2% of lung cancers. Atypical carcinoid is the rarest of the NE lung tumors, accounting for approximately 0.1–0.2% of lung cancers [15].

A historical perspective of pulmonary neuroendocrine (NE) lung tumors provides useful insights into some of the classification problems. Small cell carcinoma and carcinoid were the first neuroendocrine tumors recognized. In 1972, atypical carcinoid (AC) was described as a more aggressive form of pulmonary carcinoid [16] and large cell neuroendocrine carcinoma (LCNEC) was defined in 1991 as a high-grade non-small cell carcinoma [6]. There have been many proposals for new terminology and diagnostic criteria for pulmonary neuroendocrine tumors, such as well, moderate, and poorly differentiated neuroendocrine carcinoma, neuroendocrine carcinoma (grade 1–3), intermediate cell neuroendocrine carcinoma, malignant carcinoid, and peripheral small cell carcinoma resembling carcinoid [17–19]. While some claim that alternative terminology solves the difficult problems of pulmonary neuroendocrine tumors, the problems are found to be the same whatever the terminology used. Furthermore, the lack of uniformity has led to a great deal of confusion in clinical practice and the literature, often making it difficult to understand what tumor types are included in some papers. It is difficult to diagnose AC and LCNEC in small biopsies or cytology, and a definitive diagnosis usually requires a surgical specimen. These tumors are also clinically problematic because the optimal therapy for AC and LCNEC is not established [15].

19.2 Neuroendocrine Cell Hyperplasia and Tumorlets

Neuroendocrine cells can be found at the base of the normal bronchial and bronchiolar respiratory epithelium as non-ciliated, round- to oval-shaped cells. They have a moderate amount of cytoplasm, round to oval nuclei with finely granular chromatin and nucleoli are small or absent [20]. They have cytoplasmic processes that rarely reach the airway lumen [21]. When these cells form of a cluster of 4–10 neuroendocrine

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Table 19.1 The spectrum of neuroendocrine (NE) proliferations and neoplasms[a]

I. *NE cell hyperplasia and tumorlets*
  A. NE cell hyperplasia
    1. NE cell hyperplasia associated with fibrosis and/or inflammation
    2. NE cell hyperplasia adjacent to carcinoid tumors
    3. Diffuse idiopathic NE cell hyperplasia with or without airway fibrosis/obstruction
  B. Tumorlets (less than 0.5 cm)
II. *Tumors with NE morphology*
  A. Typical carcinoid (0.5 cm or larger)
  B. Atypical carcinoid
  C. Large cell neuroendocrine carcinoma
    Combined large cell neuroendocrine carcinoma[b]
  D. Small cell carcinoma
    Combined small cell carcinoma[b]
III. *Non-small cell carcinomas with NE differentiation (NED)*
IV. *Other tumors with NE properties*
  A. Pulmonary blastoma
  B. Primitive neuroectodermal tumor
  C. Desmoplastic round cell tumor
  D. Carcinomas with rhabdoid phenotype
  E. Paraganglioma

[a]Modified from reference [88]
[b]The histologic type of the other component of non-small cell carcinoma should be specified

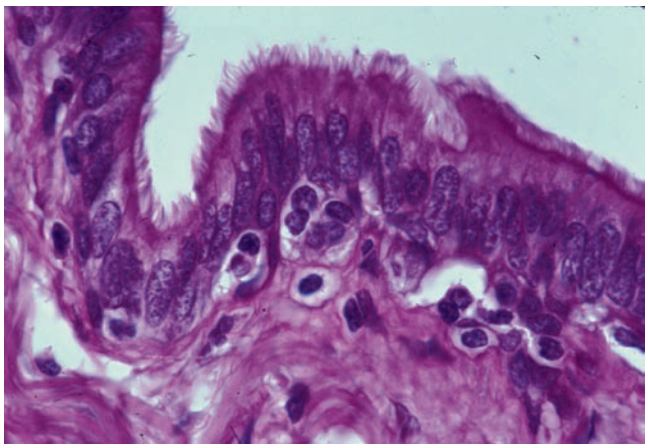

Fig. 19.1 Neuroendocrine body. There is a cluster of neuroendocrine cells situated in the base of the respiratory mucosa. The cells have eosinophilic to clear cytoplasm and finely granular nuclear chromatin

cells (Fig. 19.1), they are called a neuroendocrine body [20]. These structures are found most commonly in the epithelium of bronchi and bronchioles, often at airway branch points [20]. Neuroendocrine cell hyperplasia can consist of either clusters of neuroendocrine cells or linear hyperplasia with neuroendocrine cells growing at the base of respiratory epithelium [22–24]. Neuroendocrine cells are recognized to play an important role in lung development, regulation of airway branching [25–29], and the repair of lung injury in conditions, such as bronchopulmonary dysplasia and cystic fibrosis through mediators such as bombesin-like peptides [29, 30]. Neuroendocrine bodies appear to function as oxygen sensors and the neuroendocrine cell hyperplasia occurs in response to chronic hypoxia [30–34]. Neuroendocrine bodies are innervated and have channels responsive to mechanical stretching forces that occur in lung growth and development [35].

Tumorlets consist of peribronchiolar, nodular aggregates of uniform, round to oval or spindle-shaped cells with moderate amounts of cytoplasm that measure less than 0.5 cm in greatest diameter. These cells are identical to the neuroendocrine cells of carcinoid tumors in their morphology, immunohistochemical expression of neuroendocrine markers, and electron microscopy. They typically represent incidental histologic findings of no clinical significance, with the exception of the rare condition of diffuse idiopathic pulmonary neuroendocrine cell hyperplasia. Tumorlets are often found in lung specimens showing bronchiectasis [36], interstitial fibrosis [37], chronic abscesses [38], or tuberculosis [39].

Neuroendocrine cell hyperplasia and tumorlets are most often seen as a reactive secondary lesion in the setting of airway inflammation and/or fibrosis [38, 40–42]. Therefore, in most cases neuroendocrine cell hyperplasia does not represent a pre-invasive condition.

19.2.1 Diffuse Idiopathic Pulmonary Neuroendocrine Cell Hyperplasia

There is a very rare condition called “diffuse idiopathic pulmonary neuroendocrine cell hyperplasia” (DIPNECH) consisting of widespread peripheral airway neuroendocrine cell hyperplasia and/or multiple tumorlets. It may present as a form of interstitial lung disease with airway obstruction due to the frequent association with bronchiolar fibrosis. Since a subset of these patients has one or more carcinoid tumors, DIPNECH is thought to represent a pre-invasive lesion for carcinoid tumors [43].

There are less than 40 cases of DIPNECH reported [22, 43–45]. Aguayo et al. reported a series of six patients with widespread pulmonary neuroendocrine cell hyperplasia, tumorlets, multiple carcinoids, and airway fibrosis. They speculated that the neuroendocrine cell proliferation was the primary lesion and that substances such as bombesin produced by the neuroendocrine cells promoted the airway fibrosis [22]. This led the WHO committee for the 1999 classification of tumors of the lung and pleura to include for the first time DIPNECH as a pre-invasive lesion for carcinoid tumors, realizing this occurs in only a very small subset of carcinoid patients [43]. Davies et al. reported 19 cases with 15 females and 4 males, and 16 non-smokers [46]. There were two major types of clinical presentation: one ($n=9$) presenting with mild

interstitial lung disease, such as symptomatic cough and/or dyspnea averaging 8.6 years prior to diagnosis and the second ($n=10$) in patients found incidentally to have pulmonary nodules on routine radiologic evaluation for another disorder, mostly cancer. Tumorlets and TC were found in nine patients. Three patients had AC and one had MEN1 syndrome. Most of these patients had a stable clinical course, but a few progressed to severe airflow obstruction [46].

Lung biopsies from patients with DIPNECH typically show extensive neuroendocrine cell hyperplasia and tumorlets (Fig. 19.2a–c). Some patients also have carcinoid tumors (Fig. 19.2a). Tumorlets may cause airway narrowing and/or obliteration (Fig. 19.2b). The neuroendocrine cells may proliferate within the respiratory mucosa in either linear arrays (Fig. 19.2c) or neuroendocrine bodies. Sometimes the airway obstruction is due to fibrosis. The surrounding lung parenchyma is generally normal.

It is not uncommon to find neuroendocrine cell hyperplasia in the mucosa of bronchioles adjacent to peripheral carcinoid tumors. Miller et al. found this in 75% of 25 peripheral carcinoid tumors [24]. They also found obliterative bronchiolar fibrosis in 32% of cases [24]. DIPNECH can be distinguished from incidental neuroendocrine cell hyperplasia and tumorlets if the histologic changes are diffused throughout the representatively sampled available lung tissue and/or if there is clinical evidence of airflow obstruction or CT evidence of air trapping or multiple nodules consistent with tumorlets and/or carcinoid tumors.

19.3 Carcinoid Tumors

19.3.1 Clinical Features

There is no marked sex pre-dilection for pulmonary carcinoids [47–51]. The mean age for TC and AC patients is 45–55 years, but they can occur at any age [47–51].

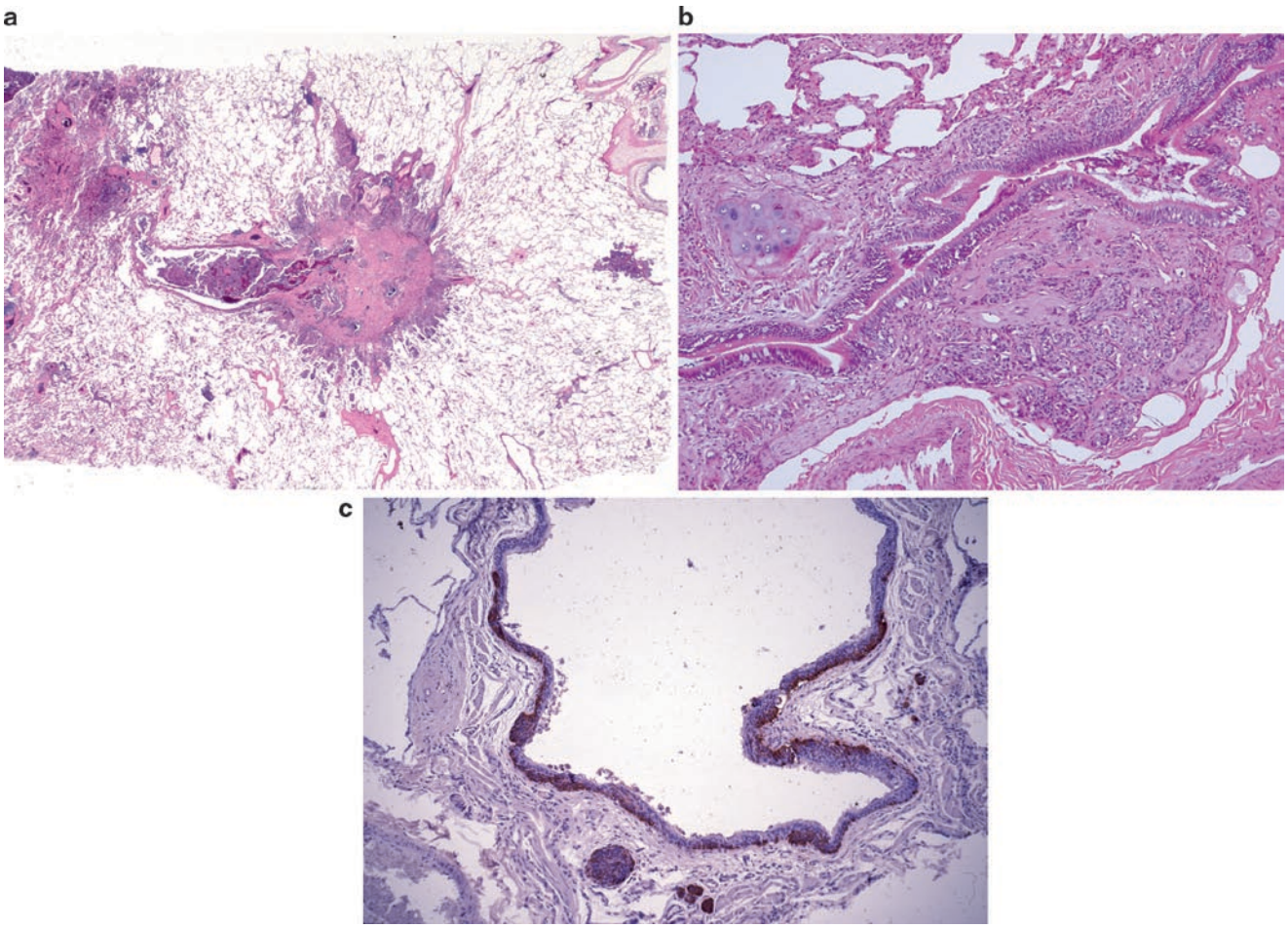

Fig. 19.2 Diffuse idiopathic neuroendocrine cell hyperplasia. (**a**) The lung parenchyma surrounding a carcinoid tumor (*center*) shows multiple tumorlets filling bronchiolar lumens. (**b**) Tumorlet consists of nodular clusters of neuroendocrine cells in a peribronchiolar location causing compression of the bronchiolar lumen. (**c**) Neuroendocrine cell hyperplasia with a linear proliferation of neuroendocrine cells and a few clusters within the base of the respiratory mucosa (Chromogranin stain)

Patients with TC and AC have similar presenting manifestations with up to 50% being asymptomatic at initial diagnosis. The most common symptoms include dyspnea, hemoptysis, cough, and post-obstructive pneumonia [49, 52]. Peripheral carcinoids are more likely to be discovered as incidental radiologic findings in an asymptomatic patient [53].

Paraneoplastic syndromes can occur including the carcinoid syndrome [49], Cushing's syndrome [54, 55], and acromegaly [56]. Bronchial carcinoids occur in approximately 5% of patients with MEN type I [57].

19.3.2 Pathologic Features

Carcinoid tumors can be located in the central or peripheral lung with up to 40% presenting as peripheral tumors. Central carcinoids frequently have an endobronchial component. Carcinoids are usually circumscribed with a tan, yellow cut surface. The average size is 2–3 cm.

The histologic features of both typical and atypical carcinoids consist of an organoid growth pattern with uniform cytologic features consisting of moderate amount of eosinophilic cytoplasm with an eosinophilic hue (Fig. 19.3a). Nuclei have finely granular chromatin, although in some atypical carcinoids it may be coarse. Nucleoli are inconspicuous in most typical carcinoids, but in atypical carcinoids they may be more prominent.

A variety of histologic patterns may occur in both AC and TC, including spindle cell [58], trabecular [6], palisading [6], glandular, follicular [6], rosette-like [6], sclerosing, clear cell, and papillary patterns [58]. Most carcinoids have multiple rather than a single histologic pattern. Tumor cells can also have an unusual cytology, such as oncocytic or melanocytic features [6, 59, 60]. Stromal ossification or calcification can occur [61–63].

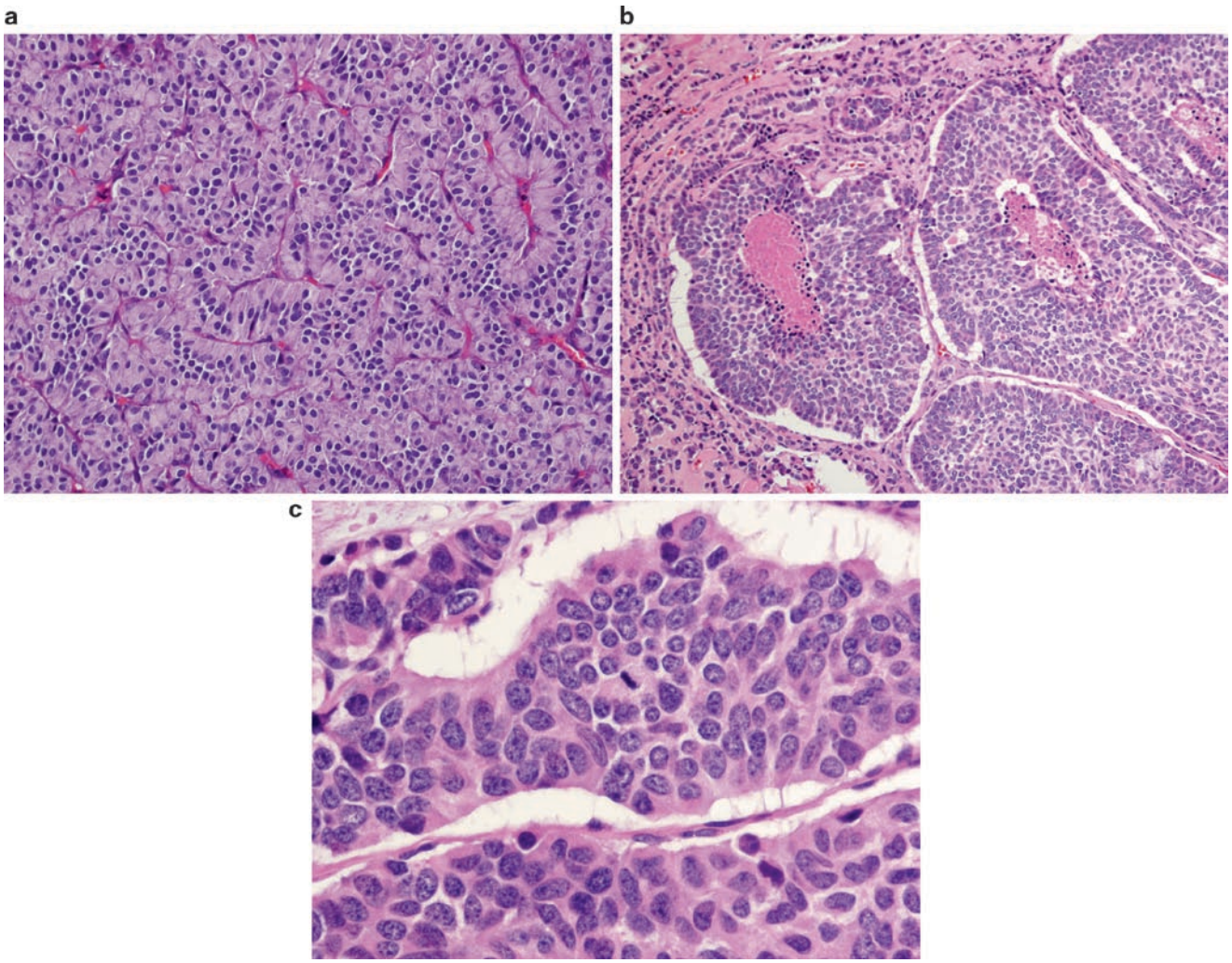

Fig. 19.3 Typical carcinoid. (**a**) This tumor shows an organoid nesting pattern of uniform cells with a moderate amount of eosinophilic cytoplasm and finely granular nuclear chromatin. (**b**) Atypical carcinoid shows a punctate focus necrosis within sheets and nests of carcinoid tumor cells. The cells have finely granular nuclear chromatin. (**c**) Atypical carcinoid shows a single mitoses (center) in one tumor cell

AC is defined as a carcinoid tumor with mitoses between 2 and 10 per 2 mm^2 area of viable tumor or the presence of necrosis (Fig. 19.3b and c) [64]. The presence of features, such as pleomorphism, vascular invasion, and increased cellularity are not as helpful in separating TC from AC (Table 19.2) [6, 16]. The necrosis in AC usually consists of small punctate foci.

Table 19.2 Criteria for diagnosis of neuroendocrine tumors [88]

Typical carcinoid
A tumor with carcinoid morphology and less than 2 mitoses per 2 mm^2 (10 HPF)[a], lacking necrosis and 0.5 cm or larger
Atypical carcinoid
A tumor with carcinoid morphology with 2–10 mitoses per 2 mm^2 (10 HPF)[a] or necrosis (often punctate)
Large cell neuroendocrine carcinoma
A tumor with a neuroendocrine morphology (organoid nesting, palisading, rosettes, trabeculae)
High mitotic rate: 11 or greater per 2 mm^2 (10 HPF)[a], median of 70 per 2 mm^2 (10 HPF)[a]
Necrosis (often large zones)
Cytologic features of a non-small cell carcinoma (NSCLC): large cell size, low nuclear to cytoplasmic ratio, vesicular or fine chromatin, and/or frequent nucleoli. Some tumors have fine nuclear chromatin and lack nucleoli, but qualify as NSCLC because of large cell size and abundant cytoplasm.
Positive immunohistochemical staining for one or more NE markers (other than neuron-specific enolase) and/or NE granules by electron microscopy.
Small cell carcinoma
Small size (generally less than the diameter of three small resting lymphocytes)
Scant cytoplasm
Nuclei: finely granular nuclear chromatin, absent or faint nucleoli
High mitotic rate (11 or greater per 2 mm^2, median of 80 per 2 mm^2)[a]
Frequent necrosis often in large zones

[a]10 HPF in a microscope with field of view of 0.2 mm^2; however, the number of HPF to reach 2 mm^2 vary depending on the field of view, see reference [64]

A recent study of frozen sections in pulmonary carcinoid tumors showed that the most common mis-classification was squamous cell carcinoma (7/66 cases, 11%) while lymphoma (12/40, 30%) and metastatic breast cancer (4/38, 13%) were frequently mistaken for carcinoid tumors [65]. According to a statistical analysis, the most helpful pathologic features in the recognition of carcinoid versus lymphoma, squamous cell carcinoma (SCC), or metastatic breast carcinoma were central location (favoring carcinoid or SCC), stromal hyalinization (favoring carcinoid), salt and pepper chromatin (favoring carcinoid), nuclear pleomorphism (favoring breast cancer and SCC), irregular nuclear membrane (favoring breast cancer, SCC, or lymphoma), and mitoses greater than 5 per 10 HPF (favoring SCC or breast cancer) [65].

19.3.3 Immunohistochemistry and Electron Microscopy

The most useful neuroendocrine markers include chromogranin, CD56, and synaptophysin. Reports on TTF-1 expression in typical and atypical carcinoids are varied, with some claiming all negative [66] and others positive expression [67, 68]. A recent report claimed that TTF-1 was expressed predominantly in peripheral carcinoids rather than central ones [67]. Most carcinoids stain for cytokeratins, but up to 20–25% may be keratin negative. Ki-67 staining shows a low proliferation rate in TC (Fig. 19.4a), usually less than 5%, while in AC it is higher, usually between 5 and 20% (Fig. 19.4b) [69–71]. The proliferation rate can be most helpful in small crushed biopsies to separate TC or AC from LCNEC or SCLC [69–71].

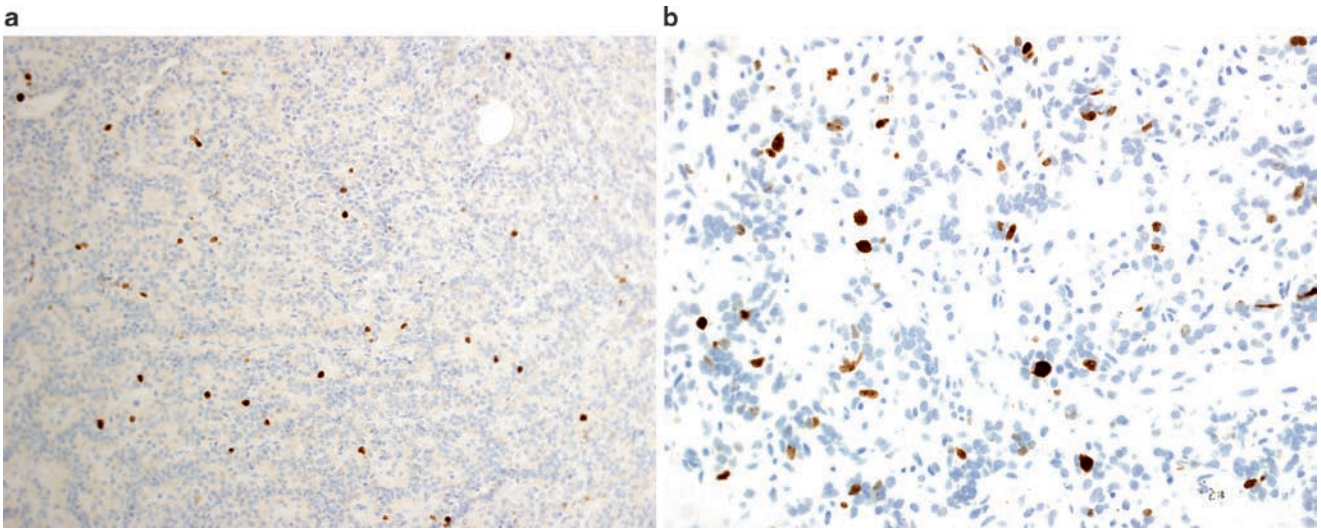

Fig. 19.4 Ki-67 in typical and atypical carcinoid. (**a**) Typical carcinoid the Ki-67 shows a very low proliferation rate with less than 5% tumor cell staining. (**b**) Atypical carcinoid the Ki-67 shows an intermediate proliferation rate with approximately 10% tumor cell staining

Dense core granules by electron microscopy are characteristic of pulmonary carcinoids, and they tend to be fewer in AC compared to TC.

19.3.4 Differential Diagnosis

The differential diagnosis includes typical versus atypical carcinoid, as well as the high-grade SCLC and LCNEC. The criteria for separation of TC and AC are reviewed in Table 19.3. In small biopsies, one can usually diagnose only carcinoid tumor. If necrosis and/or mitoses are seen, one might suspect AC. However, the distinction between TC and AC usually requires a surgical lung biopsy because in small biopsies or cytology specimens, it is difficult to determine the level of mitotic activity and to identify the characteristic punctuate necrosis. In small biopsies, the same crush artifact seen in SCLC or LCNEC can be seen in carcinoid tumors. In small biopsies, identification of mitotic activity can be difficult. In such cases, Ki-67 can be helpful because it typically shows a high proliferation rate compared to TC or AC. In resected specimens, the high-grade tumors in surgical specimens can be distinguished by their high mitotic rate. Also depending on the growth pattern or cytology of the carcinoid tumor, there is a broad list of considerations.

There are many other tumors that can be confused with carcinoid tumors, including smooth muscle tumors, sclerosing hemangioma, mucoepidermoid carcinoma, solitary fibrous tumor, metastatic breast carcinoma, and metastatic melanoma. Because carcinoid tumors typically show distinctive morphologic features including a moderate amount of eosinophilic cytoplasm with finely granular nuclear chromatin, and they usually express positive staining for neuroendocrine markers or dense core granules by EM, the differential diagnosis can usually be sorted out with immunohistochemistry.

19.3.5 Treatment and Prognosis

Surgical resection is the primary treatment for pulmonary carcinoids [49, 72–75]. Patients with TC have an excellent prognosis and rarely die of tumor [49, 76]. Lobectomy is the treatment of choice in most cases, particularly central tumors. Lung sparing procedures, such as segmentectomy, wedge excision, bronchial sleeve resection, or endoscopic resection with or without laser removal may be possible in some cases. Thomas et al. reported a series of 23 typical and 11 atypical carcinoids that presented with lymph node metastases [77]. In this study, most patients with TC and lymph node metastases had a favorable prognosis.

The 5-year survival for AC is 61–88% which is significantly reduced compared to that for TC (92–100%) [47, 52, 78–82]. At presentation, lymph node metastases are present in 4–14% of TC and 35–64% of AC. The 5-year survival for AC is 61–88% which is significantly reduced compared to that for TC (92–100%). Although the sixth edition of the AJCC and UICC did not formally accept that TNM staging applies to carcinoid tumors [83–86], TNM are recommended for pulmonary carcinoids in the seventh edition of UICC/AJCC TNM staging system [87].

These tumors are relatively resistant to chemotherapy and radiation therapy; therefore, when possible, metastatic disease is sometimes best managed surgically. There is no proven optimal therapy for metastatic unresectable TC or AC.

19.4 Large Cell Neuroendocrine Carcinoma

LCNEC is a high-grade non-small cell neuroendocrine carcinoma that is now better defined since its adoption by the WHO classification in 1999 and 2004 [43, 88, 89]. However, historically this tumor has been lumped with other tumors, such as atypical carcinoid, large cell carcinoma with

Table 19.3 Typical and atypical carcinoid: distinguishing features

Histologic or clinical feature	Typical carcinoid	Atypical carcinoid
Histologic patterns: Organoid, trabecular, palisading, and spindle cell	Characteristic	Characteristic
Mitoses	<2 per 2 mm^2	2–10 per 2 mm^2
Necrosis	Absent or secondary to infarct or prior biopsy	Characteristic, usually focal or punctate
Nuclear pleomorphism, hyperchromatism	Usually absent, not sufficient by itself for diagnosis of atypical carcinoid	Often present
Regional lymph node metastases at presentation	4–14%	35–64%
Distant metastases at presentation	Rare	20%
Ki-67	1–5%	5–20%
5-year survival	92–100%	61–88%
10-year survival	75–93%	35–67%

neuroendocrine morphology, or combined small cell carcinoma/large cell carcinoma [4, 90–92].

LCNEC was classified as a variant of large cell carcinoma in the 1999 and 2004 WHO classifications [43, 88]. With respect to neuroendocrine differentiation, there are four major categories of large cell carcinomas: (1) LCNEC with neuroendocrine features by light microscopy as well as immunohistochemistry and/or electron microscopy, (2) large cell carcinoma with neuroendocrine morphology (LCNEM) with neuroendocrine morphology but no neuroendocrine differentiation by electron microscopy or immunohistochemistry, (3) large cell carcinomas with neuroendocrine differentiation (LCC-NED) with no neuroendocrine morphology but neuroendocrine differentiation by immunohistochemistry or electron microscopy, and (4) classic large cell carcinoma (LCC) that lacks both neuroendocrine morphology and neuroendocrine differentiation by special studies [6, 43].

19.4.1 Clinical Features

LCNEC accounts for approximately 3% of surgically resected lung cancers (range 1–5%) [7, 89, 93]. Virtually all patients are cigarette smokers and most are heavy smokers with greater than a 50 pack-year history of smoking [6]. There is a strong male predominance that probably reflects a predominance of smoking in male compared to females.

LCNEC patients have a median age of 62 years (range 33–87 years) [7]. It is uncommon to encounter ectopic hormone production and paraneoplastic syndromes [7, 94, 95]. Most patients present with chest pain, followed by hemoptysis, dyspnea, cough, fever, and weight loss with up to 24% of patients being asymptomatic [92]. Oshiro et al. reported CT findings in 28 patients where most tumors were in the lung periphery (84%) and the upper lobes (63%) [96]. Other findings included endobronchial growth in 5% of cases, obstructive pneumonia in 8%, and pleural effusion in 24% [96].

19.4.2 Pathology

LCNEC are usually large peripheral tumors (66–100%) [75, 96, 97]. The average size is 3–4.0 cm with a range from 0.9 to 12 cm [64, 75, 96–98]. The tumor is typically circumscribed with a necrotic, tan-red cut surface. Larger tumors may show extensive necrosis [96].

The diagnostic criteria for LCNEC are (1) neuroendocrine morphology with organoid nesting, palisading, or rosette-like structures (Fig. 19.5a), (2) high mitotic rate greater than 10 mitoses per 2 mm^2 (average 60–80 mitoses per 2 mm^2), (3) non-small cell cytologic features including large cell size, low nuclear/cytoplasmic ratio, nucleoli, or vesicular chromatin, and (Fig. 19.5b) (4) neuroendocrine differentiation by immunohistochemistry with antibodies, such as chromogranin, CD56 or synaptophysin or electron microscopy [64, 88].

Combined LCNEC consists of a LCNEC with components of adenocarcinoma, squamous cell carcinoma, giant cell carcinoma, and/or spindle cell carcinoma [64, 88]. Most often this represents a component of adenocarcinoma, but squamous cell, giant cell, or spindle cell carcinoma can be present. If there is a component of SCLC, the tumor becomes a combined SCLC and LCNEC.

It is very difficult to diagnose LCNEC based on small biopsies or cytology. This is because of the problems in recognizing the neuroendocrine morphologic pattern and demonstrating neuroendocrine differentiation by immunohistochemistry in small pieces of tissue. Therefore, in the vast majority of cases a definite diagnosis of SCLC will require a surgical lung biopsy.

Similarly, it is difficult to diagnose LCNEC based on cytology specimens; however, several groups have addressed this subject [99–105]. The difficulty in making this diagnosis is reflected by the study of Kakinuma et al. where the diagnosis of LCNEC was not made in any of the 20 cases that they reported [101]. Instead the most common diagnosis rendered was carcinoma, histologic type undetermined, or, less often, squamous cell carcinoma, atypical carcinoid, small cell carcinoma, and large cell carcinoma [101].

19.4.3 Immunohistochemistry/Electron Microscopy

The diagnosis of LCNEC requires documentation of neuroendocrine differentiation by immunohistochemistry or electron microscopy [64, 88]. Cytokeratin antibodies, such as AE1/AE3 and CAM5.2, are typically positive [97, 106]. It is best to use a panel of neuroendocrine markers where immunohistochemical staining is usually positive for chromogranin (55–82%), CD56/NCAM (73–100%) (Fig. 19.5c), and synaptophysin (40–91%) [6, 95, 107, 108]. Staining for TTF-1 is positive in 41–75% of cases [66, 106, 109]. There is a high proliferation rate with Ki-67 staining 50–100% of tumor cells (Fig. 19.5d).

19.4.4 Differential Diagnosis

One of the most important advances in recognition of LCNEC was its distinction from the intermediate grade AC which has a much better prognosis and different biologic behavior.

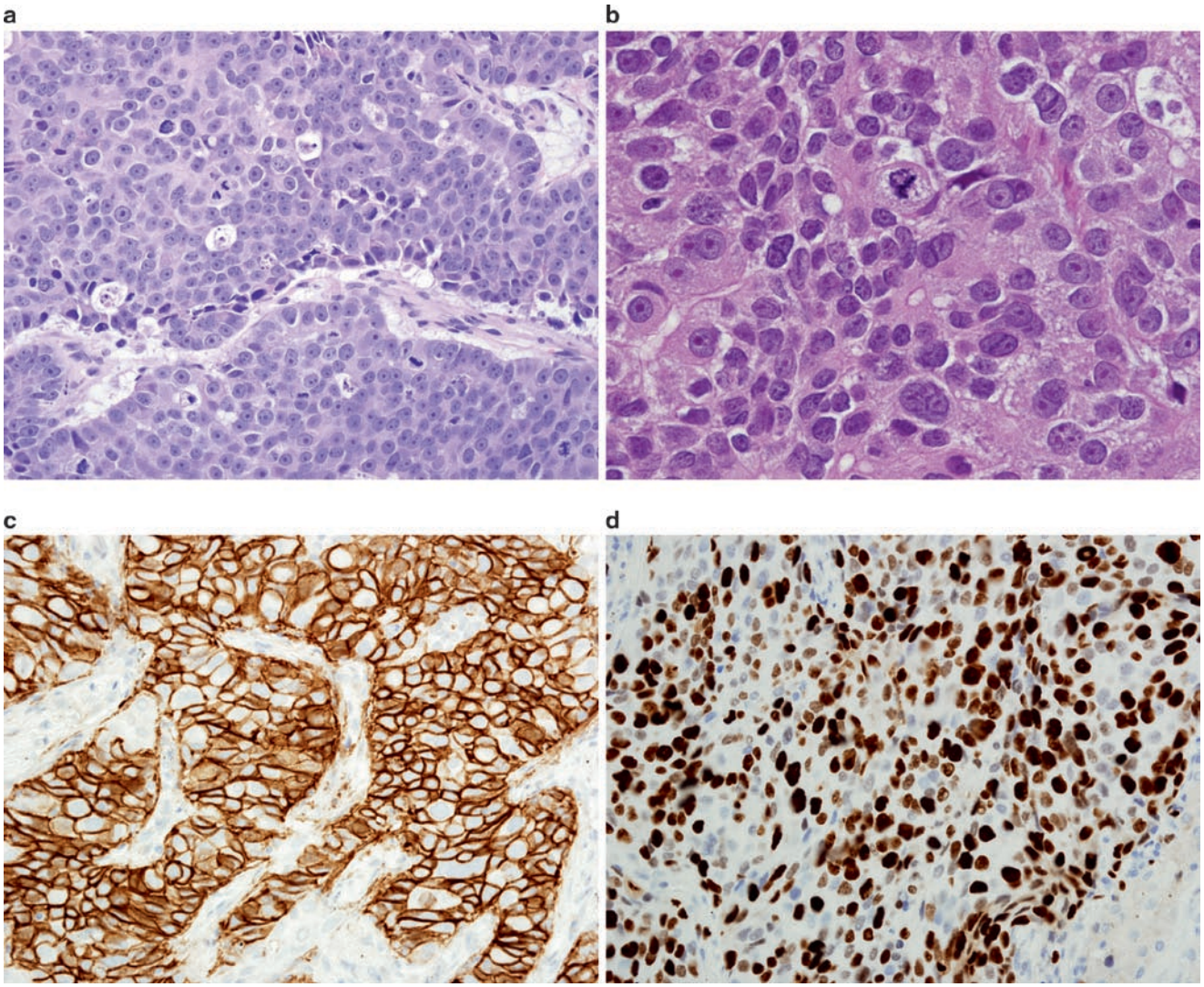

Fig. 19.5 Large cell neuroendocrine carcinoma. (**a**) The tumor grows in organoid nests with peripheral palisading, rosette-like structures and prominent mitoses. (**b**) The tumor cells have abundant cytoplasm, prominent nucleoli, and an atypical mitosis. (**c**) CD56 stains many of the tumor cells. (**d**) Ki-67 shows a high proliferation rate with approximately 70% tumor cells staining

The most useful criterion for distinguishing AC from LCNEC is the mitotic activity [6]. AC should have only upper to 10 mitoses per 2 mm^2 [88]. However, LCNEC should have a mitotic count of 11 or more per 2 mm^2 but mitotic counts typically range between 50 and 100 mitoses per 10 HPF [88]. The extent of necrosis in most LCNEC is greater than in AC where it usually consists of punctate foci. The nuclear chromatin of AC is usually finely granular, in contrast to LCNEC where it is usually vesicular or coarse.

In addition, the differential diagnosis of LCNEC includes SCLC, large cell carcinoma, large cell carcinoma with neuroendocrine differentiation (LCC-ND), large cell carcinoma with neuroendocrine morphology (LCC-NEM), and classical large cell carcinoma (LCC) without neuroendocrine features. The distinction of LCNEC from SCLC requires consideration of multiple histologic features, such as cell size, nucleoli, chromatin pattern, and nuclear/cytoplasmic ratio, rather than a single criterion (Table 19.4). Fixation or frozen section artifacts can complicate this distinction. The most important stain is a good quality hematoxylin and eosin-stained section. If histologic sections are too thick or over stained, it can be difficult to see the cellular and nuclear detail required to make an accurate diagnosis. The difficulty in making this distinction is reflected in the retrospective review of a group of previously diagnosed SCLC where up to 44% of cases were reclassified as LCNEC [95, 110].

In a reproducibility study among five pathologists, 18% of LCNEC were called SCLC and 4% AC [110]. The fewest disagreements were for SCLC, where 4% were called AC and 4% LCNEC. However, overall kappa values were mostly between 0.70 and 0.77 or substantial, with a few kappa values at 0.60 which is at the upper limit of moderate [110].

Table 19.4 Light microscopic criteria for distinguishing small cell carcinoma and large cell carcinoma[b]

Histologic feature	Small cell carcinoma	Large cell carcinoma[a]
Cell size	Smaller (less than diameter of 3 lymphocytes)	Larger
Nuclear/cytoplasmic ratio	Higher	Lower
Nuclear chromatin	Finely granular, uniform	Coarsely granular or vesicular Less uniform
Nucleoli	Absent or faint	Often (not always) present May be prominent or faint
Nuclear molding	Characteristic	Uncharacteristic
Fusiform shape	Common	Uncommon
Polygonal shape with ample pink cytoplasm	Uncharacteristic	Characteristic
Nuclear smear	Frequent	Uncommon
Basophilic staining of vessels and stroma	Occasional	Rare

[a]Modified from reference [143]
[b]Includes large cell neuroendocrine carcinoma

19.4.4.1 Large Cell Carcinoma with Neuroendocrine Morphology

This tumor was first mentioned in the 1999 WHO classification where it was recognized that some large cell carcinomas have neuroendocrine morphology (LCC-NEM), but lack positive staining with neuroendocrine markers [43]. Since these cases are very rare, there is little information available regarding the clinical characteristics of these patients [92, 98, 111]. However, the existing data suggests that the clinical features, such as age, gender pre-dilection, smoking history stage distribution, and survival are very similar to LCNEC [92, 98, 111]. For example, Iyoda et al. found that LCC-NEM had similar clinical properties like LCNEC, with the exception that LCC-NEM had a more significantly elevated serum tissue polypeptide antigen (TPA) compared to LCNEC, and a higher LDH than classic large cell carcinomas [98].

19.4.4.2 Non-small Cell Carcinomas with Neuroendocrine Differentiation

NSCLC with neuroendocrine differentiation (*NSCLC-ND*) (Table 19.1) consists of lung carcinomas, which do not show neuroendocrine morphology by light microscopy, but have neuroendocrine differentiation by immunohistochemistry and/or ultrastructure. Immunohistochemistry will show neuroendocrine differentiation in 10–20% of squamous cell carcinomas, adenocarcinomas, and large cell carcinomas [112]. It is seen most often in adenocarcinomas. The data do not show any consistent clinical significance either with regard to prognosis or responsiveness to chemotherapy [93, 113–129].

19.4.5 Treatment and Prognosis

LCNEC patients have an aggressive clinical course with overall 5-year survivals ranging from 15 to 57%. The reported variation in survival is probably due to differences in distribution of lower versus higher stage, and the thoroughness of the approach to operative staging. It is likely that the favorable survival data stage for the stage observed in some series can be attributed to the careful approach to surgical staging, such as systematic nodal dissection [92].

Survival for LCNEC is significantly worse than that for other non-small cell carcinomas. According to Jiang et al., LCNEC patients had 58.8% and 44.8% one- and five-year survival, respectively, which was a significantly worse prognosis than for patients with other non-small cell carcinomas ($p=0.046$) [107]. Iyoda also found that the survival for LCNEC was significantly worse than that for classical large cell carcinoma [98]. Takei et al. compared survival in Stage I patients with LCNEC, poorly differentiated NSCLC, and LCC were 67%, 88% and 92% and found significant differences between LCNEC and NSCLC ($p=0.003$), LCNEC and LCC ($p=0.03$) but not between LCNEC and SCLC [95].

There is little data on the efficacy of chemotherapy for LCNEC. The major question is whether LCNEC should be treated similar to small cell lung cancer because of the neuroendocrine features. While the aggressive clinical course and tendency to metastasize is similar to SCLC, it remains to be proven whether LCNEC is also chemosensitive. In recent years, several studies have demonstrated clinical response to cisplatin-based chemotherapeutic regimens similar to those used for SCLC [130–132]. However, these are retrospective studies of small numbers of patients who received adjuvant therapy following surgery and are all retrospective series. The study by Rossi et al. showed that patients who received platinum-etoposide based chemotherapy had the best survival in both the adjuvant and metastatic setting [131]. A small series reported by Filosso and colleagues suggested that octreotide may be effective alone or in combination with radiation therapy when given as an adjunctive treatment [133].

There is too little data available on radiation to know whether it is effective in LCNEC or not [90, 95, 98, 134, 135].

Further investigation is needed regarding the spectrum of clinical and pathologic features of LCNEC and optimal therapeutic approach. Hopefully future work will further define the differences in survival and response to therapy for LCNEC compared to atypical carcinoid, LCNEM, LCC-NE, LCC, and SCLC.

19.5 Small Cell Carcinoma

19.5.1 Clinical Features

Small cell lung cancer (SCLC) is the most common pulmonary neuroendocrine tumor accounting for an estimated 28,000 of the 215,020 lung cancer cases diagnosed in the United States in 2008 [136]. According to the United States National Cancer Institute's Surveillance, Epidemiologic, and End Results (SEER) database, the proportion of SCLC cases among all lung cancers in the U.S. decreased from 17 to 13% in the last 30 years [137].

Virtually all SCLC patients are cigarette smokers [138]. In fact, if the diagnosis of SCLC is suggested in a nonsmoker, the pathology diagnosis should be carefully reevaluated. Clinically, SCLC is a tumor that grows rapidly and metastasizes early [138]. The presenting manifestations of SCLC can be divided into four categories: constitutional, pulmonary, the result of extrathoracic spread, or due to paraneoplastic disorders [138]. Typical symptoms include fatigue, cough, dyspnea, decreased appetite, weight loss, pain, and hemoptysis. The typical radiologic finding is a large central mass invading or compressing the mediastinum with hilar or mediastinal adenopathy. In 10% of patients, superior vena cava obstruction is found at presentation [138]. Rarely SCLC presents as a solitary pulmonary nodule [139]. Most patients with SCLC have metastases at diagnosis involving sites such as bone, brain, liver, and adrenals [138]. The paraneoplastic syndromes associated with SCLC include the syndrome of inappropriate antidiuretic hormone (SIADH), Cushing's syndrome, or neurologic paraneoplastic syndromes, such as autoimmune neuropathies and encephalomyelitis that probably have autoimmune mechanisms [138].

Because of its tendency to metastasize early, a two-stage system of limited versus extensive stage disease, apart from TNM staging, has been recommended by the Veterans' Administration Lung Study Group (VALSG) for SCLC [140]. Approximately, two-thirds of patients with SCLC have extensive disease at diagnosis, and one-third have limited-stage disease [137]. According to the VALSG system, limited stage consists of tumor confined to one hemithorax that can be "encompassed" in a "tolerable" radiation field. Less than 10% of patients, who have tumor only involving the lung, are surgical candidates. Recently, an analysis of a large database of over 8,000 patients demonstrated that TNM staging is effective for SCLC and the use of TNM staging will be recommended in the upcoming seventh edition [141].

19.5.2 Pathology

Because most patients are unresectable at presentation, the majority of specimens obtained for the diagnosis of SCLC are small, such as bronchoscopic biopsies, fine needle aspirates, core biopsies, and cytology. The diagnosis can readily be established based on these specimens in the vast majority of cases. While some of the description in the 2004 WHO classification deals with issues involving surgical resection specimens [88], most of the criteria are applicable to small biopsies as well.

When surgically resected, the diagnosis of SCLC usually has not been established prior to surgery and the tumor consists of a peripheral nodule measuring 2–4 cm in size. The tumor is usually circumscribed with a tan, necrotic cut surface.

The diagnosis of SCLC is based primarily on light microscopy (Fig. 19.6a). Necrosis is common, frequently with large areas. Although there is no absolute criterion for cell size, in general, the tumor cells measure less than the diameter of three small resting lymphocytes. Tumor cells are usually round to fusiform with scant cytoplasm. Nuclear chromatin is finely granular and nucleoli are inconspicuous or absent [88, 142]. There is usually a high mitotic rate, averaging 60–80 per 2 mm^2. Although this may be more difficult to appreciate in small biopsy specimens, usually frequent mitoses can be detected. The crush artifact and tumor streaming seen often in small transbronchial or mediastinal biopsy specimens can complicate pathologic interpretation. These artifacts can also occur with non-small cell lung cancer (NSCLC), lymphoma, carcinoid, and chronic inflammation. The cells of SCLC appear larger than in small biopsies in surgically resected specimens where the tumor is better fixed [88, 142, 143]. SCLC are called combined SCLC when there is also a component of NSCLC, such as adenocarcinoma, squamous cell carcinoma, large cell carcinoma, spindle cell carcinoma, and giant cell carcinoma. In this setting, the specific histology of the non-small cell component should also be mentioned [88, 142]. In resected specimens, combined SCLC may occur in up to 28% of cases [88, 142]. Although there is no percentage requirement for the components of adenocarcinoma, squamous cell, spindle cell, or giant cell carcinoma when these result in a combined SCLC, in order to diagnose combined SCLC and large cell carcinoma, the large cell carcinoma component must comprise at least 10% of the overall tumor [88, 142].

The most important special stain for a SCLC diagnosis is a good quality H&E stain that is not too thick or over stained.

Immunohistochemistry may be helpful but if the histologic features are classic, it may not be needed. Since virtually all SCLC stain for keratin, a pancytokeratin antibody such as AE1/AE3 is useful to confirm if the tumor is a carcinoma. If keratin is negative, stains for other tumors such as lymphoma, including CD45 and CD20, or stains for primitive neuroectodermal tumors (PNET), such as CD99, may be helpful. The most useful neuroendocrine markers include CD56 (Fig. 19.6b), chromogranin, and synaptophysin, which are best used as a panel. TTF-1 expression is found in 70–80% of SCLC (Fig. 19.6c) [66, 106, 109]. SCLC must be separated from other NSCLC, carcinoid tumors, malignant lymphoma, and sarcomas such as PNET. This differential diagnosis can be very difficult in small, crushed biopsy specimens. Since cytology is often obtained at the time of bronchoscopic biopsy and SCLC shows very characteristic cytologic features, comparison of biopsy material with the cytology specimen can be very helpful. In particular, large cell neuroendocrine carcinoma and the basaloid variant of large cell carcinoma can be difficult to distinguish from SCLC. SCLC can be distinguished from carcinoids by a high proliferation rate of 80–100% with Ki-67 (Fig. 19.6d).

In approximately 5% of the cases, SCLC can be difficult even for expert lung cancer pathologists to separate from non-small cell carcinoma [110, 144, 145]. The best approach for these cases may require special scrutiny using a consensus approach, as suggested by other pathologists. If a consensus diagnosis cannot be reached, it may be appropriate to refer the case for extramural consultation. Immunohistochemical markers can be of assistance in crushed specimens, as SCLC may demonstrate positive staining for cytokeratin, chromogranin, CD56, synaptophysin, TTF-1, and a high proliferation

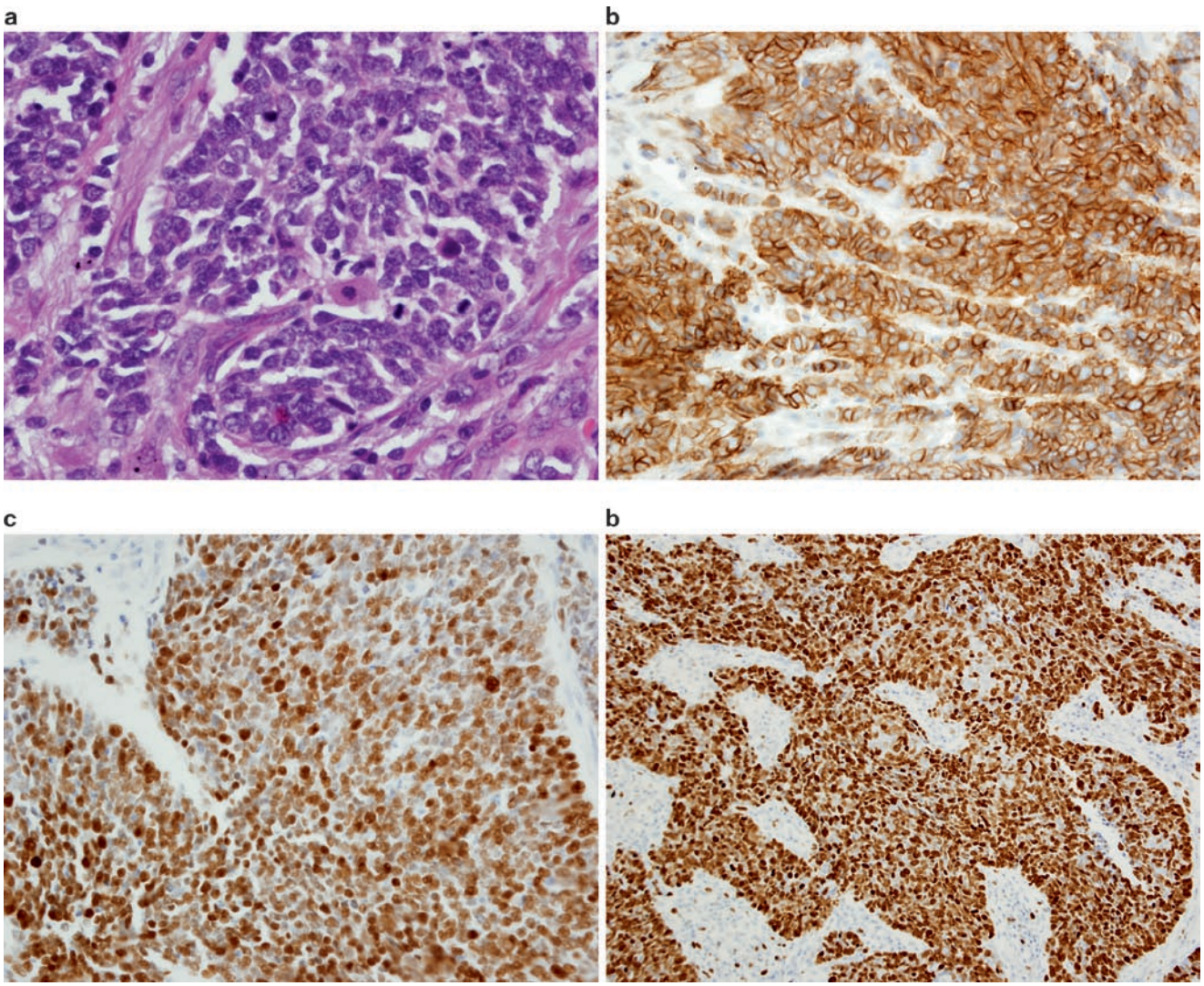

Fig. 19.6 Small cell carcinoma. (**a**) This tumor consists of dense sheets of small cells with scant cytoplasm, finely granular nuclear chromatin, frequent mitoses, and nucleoli are inconspicuous or absent. (**b**) CD56 is positive with a membranous pattern in this SCLC. (**c**) TTF-1 shows diffuse positive nuclear staining. (**d**) Ki-67 shows a high proliferation rate with almost 100% tumor cell staining

index with Ki-67 [8]. However, some preserved tumor cells with characteristic morphology should be seen on light microscopy to confirm the diagnosis. Up to 10% of SCLC may be negative for all neuroendocrine markers if a panel of antibodies including CD56 is utilized, so if all other features are present the diagnosis of SCLC should not be avoided [146]. Since TTF-1 can be positive in extrapulmonary small cell carcinomas, it should not be used to determine the primary site of small cell carcinomas [147].

19.5.3 Differential Diagnosis

The differential diagnosis for SCLC has been addressed with regard to LCNEC, other NSCLC, and carcinoids. In addition, the use of immunohistochemistry to distinguish SCLC from lymphoma and PNET has been mentioned. Problems in small crushed biopsies have also been discussed. While most cases can be diagnosed with routine hematoxylin and eosin-stained sections, making sure a good quality H&E stain that is not too thick or over stained is one of the most important steps to a correct diagnosis.

19.5.4 Treatment and Prognosis

Survival for SCLC is poor with the Surveillance, Epidemiology, and End Results program database, reporting an overall survival at 2, 3, and 5 years of 12%, 7%, and 5%, respectively [148]. Poor prognostic factors include performance status, Cushing's syndrome, continued smoking, and metastases to sites, such as the liver, brain, bone marrow, and bone [138]. Female gender has been associated with improved survival and response to therapy [138].

The mainstay of treatment for SCLC is a combination chemotherapy, typically with etoposide plus either cisplatin or carboplatin [138]. Patients with limited-stage disease usually are given chemotherapy concurrently with radiation.

19.6 Molecular Changes in Pulmonary Neuroendocrine Tumors

Genetic studies reveal important molecular differences among the spectrum of neuroendocrine lung tumors. In general, LCNEC and SCLC show frequent genetic changes with fewer seen in the carcinoids. A limited number of genetic markers demonstrate significant differences between TC and AC or LCNEC and SCLC, but these findings support the concept that these tumors should be classified separately.

Onuki et al. demonstrated that the high-grade LCNEC and SCLC had a significantly higher frequency of loss of heterozygosity (LOH) for 3p, RB, 5q21, 9p, and p53 than in the carcinoids [11]. Significantly more frequent 5q21 LOH was found in SCLC compared to LCNEC as well as in the high-grade carcinomas compared to carcinoids. In addition, there were increasing percentages of p53 abnormalities by immunohistochemistry, LOH, and mutation analysis from TC to AC and the high-grade SCLC and LCNEC (Fig. 19.7) [11]. No p53 mutations were found in TC, with 25% in AC,

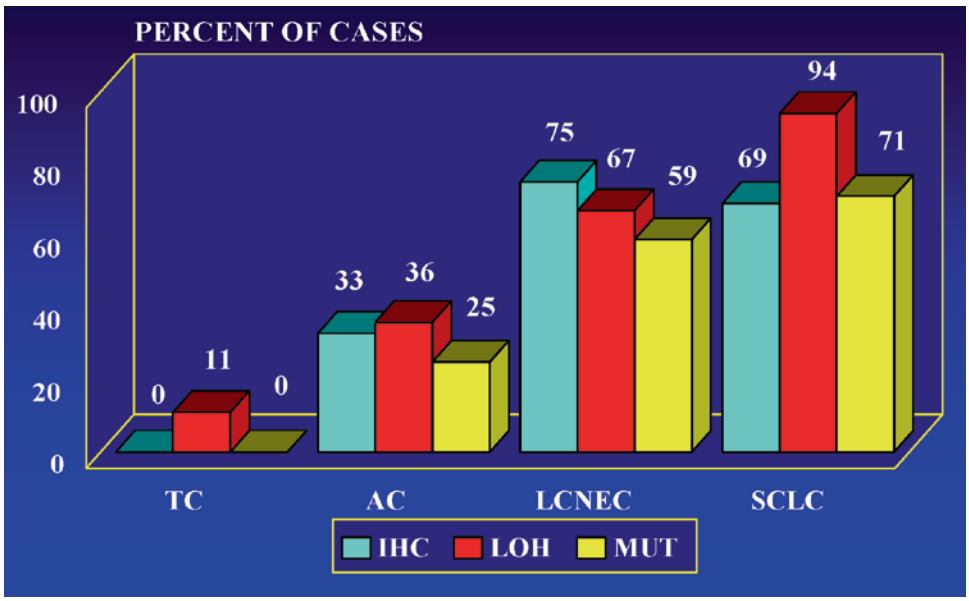

Fig. 19.7 p53 abnormalities in pulmonary neuroendocrine tumors by immunohistochemistry, loss of heterozygosity, and mutation analysis. There are frequent abnormalities in the high-grade SCLC and LCNEC, intermediate levels in AC and virtually none in TC. Data abstracted from Onuki et al. [11]

59% in LCNEC, and 71% in SCLC. These results are similar to the data reported by others in high-grade neuroendocrine carcinomas with p53 expression ranging between 40 and 86% and p53 mutations from 27 to 59% [5, 12, 70, 149–151]. Interestingly, Onuki et al. found that 58% of the point mutations found in high-grade neuroendocrine tumors were G:C to T:A or other transversions [11]. The G:C to T:A transversions are associated with carcinogens found in cigarette smoke, consistent with the high frequency of heavy cigarette smoking in LCNEC and SCLC patients [152]. However, these transversions were not found in the mutations found in AC, corresponding with the fact that AC patients have significantly less smoking histories compared to LCNEC and SCLC [11]. A single K-RAS mutation was found in one LCNEC [11].

The $P16^{INK4}$/cyclin D1/Rb pathway is involved in the regulation of G1 arrest in the cell cycle and is frequently affected in NE tumors [153, 154]. Rb loss is found in a high percentage of SCLC and LCNEC but not in TC, and in only 21% of AC. There is an inverse relationship between Rb and P16 in the high-grade tumors and a direct relationship between cyclin D1 and Rb in all tumors indicates that p16 and cyclin D1 act exclusively on the Rb pathway for cell cycle regulation [153]. Igarashi also demonstrated an overexpression of cyclin B1 in a high percentage of LCNEC and SCLC. These data demonstrate that loss of Rb is the most frequent mechanism of Rb cell cycle pathway deregulation in LCNEC and SCLC [153, 154]. The frequent expression of cyclin B1 in LCNEC and SCLC is consistent with the concept that regulation of cyclin B1 expression and G2/M arrest are consistently compromised in high-grade neuroendocrine carcinomas [154].

Study of apoptosis show that LCNEC has a high index (1.3–6.8%) of apoptosis compared to carcinoids that have a variable apoptotic index and SCLC that have almost no apoptosis [5]. In contrast to TC and AC that have predominant Bax expression, LCNEC and SCLC have a high Bcl2/bax ratio [5, 155]. These findings are consistent with the concept that LCNEC have a high rate of cell division that could be worsened by abrogation of cell death which is favored by high Bcl2 and low Bax levels; this could result in a short doubling time and tumor aggressiveness [5].

Several studies have evaluated the C-kit protein expression in pulmonary neuroendocrine tumors. Pelosi et al. found frequent positive membranous/cytoplasmic expression in the high-grade tumors with 77/44% of LCNEC, 70/67% of SCLC, and much less staining with 7/5% in carcinoids [156]. C-kit staining was demonstrated in 55% and 61% of LCNEC by Araki et al. and Casali et al., respectively [157, 158]. A significantly worse prognosis ($p=0.046$) as well as a higher rate of recurrence (0.037) was found by Casali et al. for patients with C-kit positive LCNEC [158]. However, with LCNEC and SCLC neither Pelosi et al. nor Araki et al. found any prognostic significance to C-kit expression [156, 157].

LOH at chromosome 11q13, the site of the MEN1 gene is found in lung carcinoids from familial MEN1 patients [159]. In addition, LOH at this locus and MEN1 gene mutations can be demonstrated in up to 36% of sporadic carcinoids, particularly AC [160]. However, MEN mutations are very rare in LCNEC and they are not found in SCLC [110, 160, 161]. In one of 13 LCNEC, Debelenko et al. found a somatic frameshift in the MEN1 gene (1226delC), that represented the first mutation observed in a tumor not typically associated with MEN1 [161]. On the other allele, neither a deletion nor a mutation was detected and wild-type mRNA sequence was expressed. This suggested that the typical two-hit mechanism of MEN1 gene inactivation had not taken place [161].

As more study is focused on molecular alterations in neuroendocrine tumors, it is hoped that it may lead to novel therapeutic approaches. As we do not have effective therapies for LCNEC, AC, or TC with metastases, understanding of the molecular changes in the entire spectrum of pulmonary neuroendocrine tumors is important. Since these tumors are so uncommon, there are few molecular studies that have examined large numbers of these rare variants. Also, since only few institutions have frozen tissue banks, most molecular studies of these tumors have been retrospective studies performed on formalin-fixed and paraffin-embedded tissue samples, limiting the type of molecular studies that can be performed.

19.7 International Registry of Pulmonary Neuroendocrine Tumors

Because of the great need for collaboration to gather sufficient numbers of the rare subtypes of pulmonary neuroendocrine tumors including TC with metastases, AC, LCNEC, and surgically resected SCLC, the International Association for the Study of Lung Cancer has developed an International Registry of Pulmonary Neuroendocrine Tumors [162]. This will provide a network of collaborations to foster research of these tumors with the hope that it will lead to the development of novel molecular targeted therapies for these tumors.

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Chapter 20
Cutaneous Neuroendocrine Tumors

Lori A. Erickson

20.1 Merkel Cell Carcinoma

20.1.1 Clinical

Merkel cell carcinoma (MCC) is an uncommon, aggressive tumor, occurring most often in the sun-damaged skin of Caucasian patients in the seventh decade, but it may also occur over a wide age range [1]. The tumor presents as a painless, solitary erythematous nodule, often with recent rapid growth [2]. The majority of tumors are presumed to be benign (cyst/acneiform lesion) clinically [2].

Merkel cell carcinomas have presented in lymph nodes with no cutaneous tumor [3, 4]. These may be primary to the lymph nodes, but cutaneous MCC have undergone complete spontaneous regression [5]. From 1986 to 2001, 1,124 cases of MCC were identified using the surveillance, epidemiology, and end results registry. The incidence of MCC has increased threefold from 0.15 cases per 100,000 in 1986 to 0.44 cases per 100,000 in 2001 [6]. It is estimated that 1,500 new cases of MCC will be diagnosed in 2008 [7].

20.1.2 Immunosuppression and Tumor Associations

Immunosuppression has been observed in 7.8% of patients with MCC [2]. Organ transplant patients have a tenfold increased incidence of MCC [8]. Patients with HIV/AIDS also have an increased risk of MCC [9]. Patients with non-Hodgkin lymphoma, multiple myeloma, and malignant melanoma are also at risk for developing MCC [10]. Chronic lymphocytic leukemia is 30-fold over-represented in patients with MCC [2]. Merkel cell carcinomas can occur in both synchronous and metachronous fashion with squamous cell carcinoma (SCC) and basal cell carcinoma (BCC), respectively [11, 12]. Merkel cell carcinoma has been histologically admixed with SCC and BCC.

20.1.3 Pathology

Grossly, MCC are solitary, non-ulcerated tumors based in the dermis measuring 1.8 cm [2] to 2.8 cm [3]. The tumor cells diffusely involve the dermis and extend into the subcutaneous adipose tissue (Fig. 20.1). The tumors can also have an organoid or trabecular growth pattern. The epidermis is usually uninvolved, but intrepidermal MCC can occur even without a dermal component. The cells are usually intermediate in size, but can appear similar to small cell carcinoma, and have stippled neuroendocrine chromatin and amphophilic cytoplasm. Mitotic figures and apoptotic cells are numerous. A review of 156 MCC found solar elastosis, tumor thickness, size, anatomic compartment, growth pattern, lymphovascular invasion, tumor-infiltrating lymphocytes, and solar elastosis to be poor prognostic features by univariate analysis [13]. By multivariate analysis, diffuse growth, depth, and lymphovascular invasion were found to be poor prognostic factors [13].

20.1.4 Immunohistochemical Profile

Merkel cell carcinomas are positive for chromogranin, synaptophysin, and cytokeratin (CK) 20 and are negative for CK7 and TTF1 [14, 15]. A characteristic perinuclear dot-like staining pattern is seen with CK20 and other low molecular weight CK. Neurofilament, CD99, FLI-1, CD117, CD23, PAX-5, Cox-2, TdT, glypican-3, and stathmin have been observed in certain cases, but are not specific to MCC [16–23]. Expression of p63 and nuclear expression of survivin have shown prognostic significance [24, 25]. Expression profiles have shown correlations between metastases and matrix

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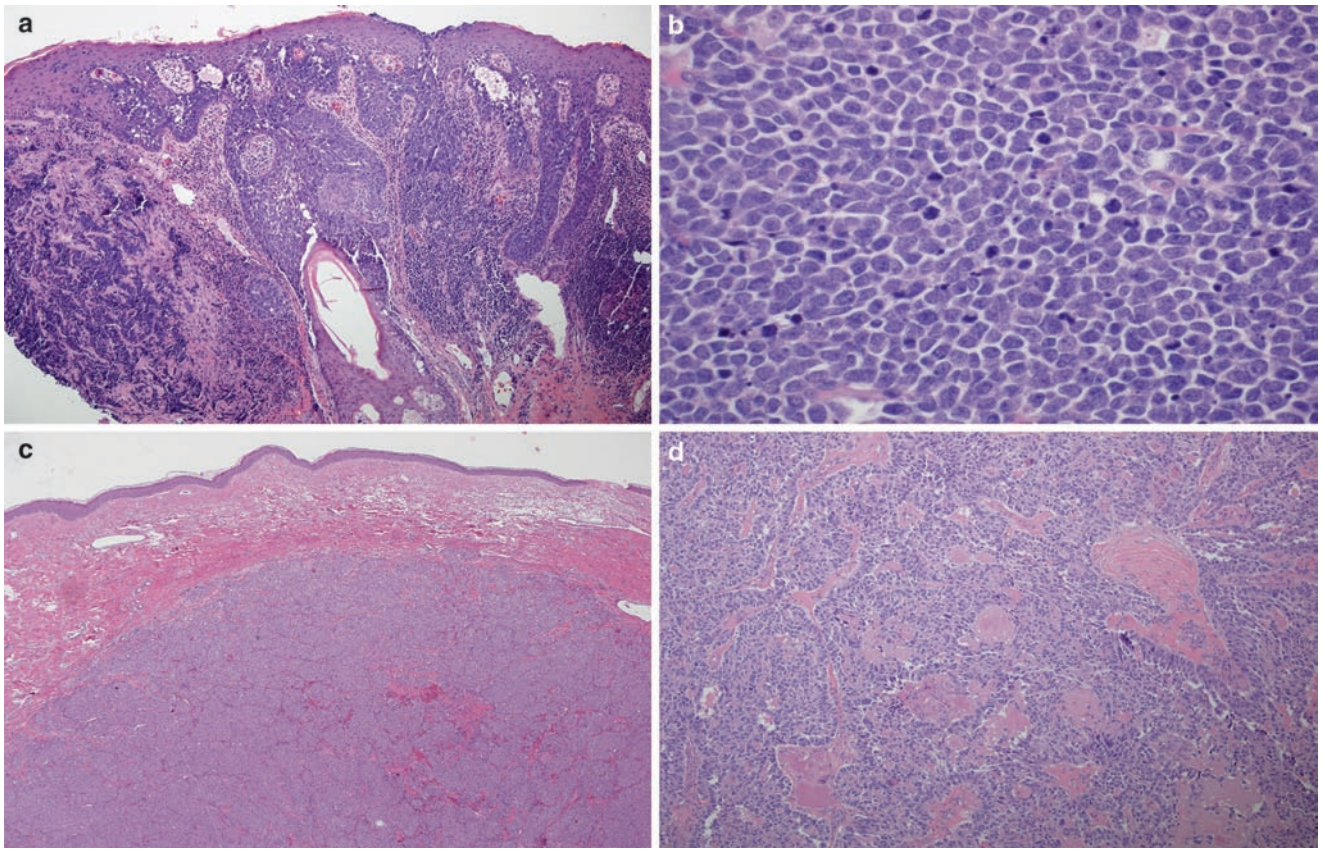

Fig. 20.1 Merkel cell carcinoma and neuroendocrine tumors metastatic to the skin. (**a**) Unusual case of Merkel cell carcinoma showing involvement of the overlying epidermis. (**b**) High power photomicrograph of diffuse growth and cytomorphology of Merkel cell carcinoma cells with stippled neuroendocrine chromatin, numerous mitoses, and apoptotic cells. (**c**) Metastatic pulmonary carcinoid tumor to the scalp. The tumor was positive for Cam5.2, cytokeratin 7, chromogranin, synaptophysin, and TTF1. (**d**) Metastatic medullary thyroid carcinoma to the skin of the neck. The tumor cells have stippled neuroendocrine chromatin. Amyloid is also present

metalloproteinase 7, tissue inhibitor of metalloproteinase 3, VEGF, P38, stromal NF-kappaB, and synaptophysin [26].

20.1.5 Merkel Cell Polyomavirus

Merkel cell polyomavirus (MCV) is a new human virus that was recently associated with MCC [27]. Investigators utilized digital transcriptome subtraction (DTS), a method they developed to identify foreign (viral) transcripts utilizing high throughput cDNA sequencing data [27, 28]. The method requires precise discrimination between human and non-human cDNA sequences as it subtracts known human sequences from the expression data, leaving non-human sequences for analysis [28]. From a cDNA library, constructed from mRNA from an MCC examined by DTS, a fusion transcript was detected between an undescribed virus T antigen and a human receptor tyrosine phosphatase [27]. The sequence of the viral genome was identified. Further studies showed that 8 of 10 MCC (as well as a corresponding metastasis) were positive for MCV sequences by PCR, while only 5 of 59 control tissues showed positivity [27]. In six of the eight positive cases, MCV DNA was integrated in the tumor genome in a clonal pattern, indicating that MCV infection and integration preceded the clonal expansion of tumor cells [27]. A study identified MCV DNA by PCR 30 of 39 (77%) formalin-fixed paraffin-embedded MCC which were confirmed by sequence analyses [29]. Thus, MCV may have an etiologic role in MCC.

20.1.6 Molecular

A recent study utilizing comparative genomic hybridization (CGH) showed a mean of 5.5 chromosome gains and 1.1 losses in 13 of 19 MCC studied [30]. The majority involved chromosomes 1, 5, and 6 and were associated with large tumors and metastases [30]. A study utilizing CGH and M-FISH found rearrangements of 1p and 3q, and a gain of 5p was the most frequent abnormality, although gains of 1q, 3q,

and 8q and losses of 3p, 10q, 13q, and 17p were also identified [31]. Trisomy 6 is a recurrent abnormality in MCC in lymph nodes [32].

Alterations of chromosome 1 are common in MCC, and deletion of chromosome 1p possibly due to a tumor suppressor gene may have a role in MCC [33–35]. Loss of both copies of chromosome 13 has been associated with an increased survival [33].

A CGH study of MCC and SCC found UVB-specific mutations in the *p53* tumor-suppressor gene and *Ha-ras* gene [36]. Loss of 3p, characteristic of SCC, was rare in MCC. 3p loss in SCC cell lines correlated with reduced expression of *FHIT* (fragile histidine triad) gene (3p14.2) and two MCC cell lines with normal 3p showed aberrant or no FHIT transcripts [36]. In addition to the common UVB-specific mutations, MCC and SCC share chromosomal imbalances suggestive of environmentally derived (e.g., UVA) oxidative damage [36]. *TP53* mis-sense and non-sense mutations were detected in 3 of 15 MCC, suggesting that *TP53* mutations may play a role in a subset of MCC [37]. One MCC cell line showed typical UVB induced C to T mutations, providing further evidence for sun exposure in the etiology of MCC [37].

Alterations in the tumor-suppressor gene *SDHD* (succinate-ubiquinone oxidoreductase subunit D), which can be mutated in paragangliomas, was identified in two of seven MCC [38]. *BRAF* T1796A mutation has not been identified in MCC [39]. Merkel cell carcinomas show frequent allelic loss of 10q23, but have a low incidence of *PTEN* mutation [40]. CD117 has been identified in MCC, but CD117 immunopositivity does not correlate with activating mutations in *KIT* and *PDGFRA* [41]. Imatinib mesylate is considered an unlikely therapy for MCC, unless activating mutations exist in other exons of these receptor kinases [41].

20.1.7 Differential Diagnosis

Merkel cell carcinoma must be distinguished from other neuroendocrine tumors, including metastatic pulmonary small cell carcinoma, atypical laryngeal carcinoid, and medullary thyroid carcinoma (Table 20.1). All of these tumors are positive for the chromogranin and synaptophysin. Merkel cell carcinomas are positive for CK20 and negative for CK7 and TTF1, and small cell lung carcinoma is positive for CK7 and TTF1 and negative for CK20 [14, 15, 42]. Both medullary thyroid carcinoma and atypical laryngeal carcinoid are positive for calcitonin, but medullary thyroid carcinoma is also positive for TTF1. Small cell carcinoma of the salivary glands has an identical immunophenotype to MCC [15]. TdT and PAX-5 can present diagnostic pitfalls as both MCC and lymphoblastic lymphoma can show staining for PAX-5 and TdT [21].

20.1.8 Treatment and Prognosis

Merkel cell carcinomas are aggressive tumors that frequently recur if incompletely excised and involve the regional lymph nodes at presentation [3, 43, 44]. Sites of distant metastases are liver, lung, brain, and bone [3, 43, 44]. Relapses and recurrences generally occur within 2 years, and most deaths within 3 years [45]. Patients with localized skin disease and negative lymph nodes have a 97% survival rate at 5 years [45]. Pathologic staging identifies metastatic disease in one-third of patients whose tumors would otherwise be clinically and radiologically understaged [46]. Adjuvant nodal therapy is beneficial with positive sentinel lymph nodes as patients who received adjuvant nodal therapy had a relapse-free survival rate of 51% at 3 years compared with 0% for patients who did not receive adjuvant therapy [46]. Radiation following resection may improve locoregional control [1]. In locally advanced or metastatic disease chemotherapy is often used, although the response is often short-lived and the impact on overall survival is uncertain [1].

20.2 Other Cutaneous Tumors with Neuroendocrine Differentiation

Neuroendocrine features have been identified in non-melanocytic skin tumors, such as BCC, sweat gland tumors, trichoblastomas, and trichofolliculomas [47]. This is an interesting finding, but does not appear prognostically significant. A few cases of large-cell neuroendocrine carcinoma of the skin with lymphoid stroma have been reported as a distinct lesion [48]. Larger series with long-term follow-up are needed to clarify this proposed entity.

Table 20.1 Differential diagnosis of Merkel cell carcinoma by immunohistochemistry

Tumor	Chromogranin	Synaptophysin	CK20	CK7	TTF1	Calcitonin	CEA
Merkel cell carcinoma	+	+	+	–	–	–	–
Small cell lung metastasis	–/+	+/–	–	–/+	+	–	–
Atypical laryngeal carcinoid	+	+	–	–	–	+	+
Medullary thyroid carcinoma	+	+	–	+	+	+	+

20.3 Cutaneous Metastatases of Neuroendocrine Tumors

20.3.1 Cutaneous Metastases of Visceral Tumors

Up to 5% of visceral carcinomas metastasize to the skin [49]. Skin metastases from visceral tumors often present in the skin near the primary tumor, but can spread to unusual sites. Breast, colon, and ovarian cancers are most common visceral carcinomas to metastasize to the skin in women, and lung and colon are most common in men [50]. Skin metastases usually occur in disseminated disease, but visceral carcinomas present in the skin in 0.8% of cases [51].

20.3.2 Neuroendocrine Tumor Metastases to the Skin

Cutaneous metastases of neuroendocrine tumors are uncommon, but they do occur (Fig. 20.1). Skin metastases of medullary thyroid carcinoma usually occur in the neck in the setting of advanced disease, but can be the presenting manifestation [52]. Medullary thyroid carcinoma needs to be differentiated from atypical laryngeal carcinoid, a tumor that can present as a cutaneous metastasis [53]. Both medullary thyroid carcinoma and atypical laryngeal carcinoid are positive for chromogranin, synaptophysin, calcitonin, and CEA [53, 54]. Thus, TTF1 is helpful as it stains medullary thyroid carcinoma but not atypical laryngeal carcinoid [53, 54].

Pulmonary neuroendocrine tumors can metastasize to the skin (Fig. 20.1). Pulmonary carcinoids are positive for chromogranin, synaptophysin, and TTF1, but negative for calcitonin [55–57]. Small cell lung carcinoma is positive for TTF1, but TTF1 is not specific for pulmonary small cell carcinomas as extrapulmonary small cell carcinomas can also be positive for TTF1 [42].

CDX2 is positive in greater than 90% of mid-gut carcinoids, but few lung, gastric, or colon carcinoids are positive for CDX2 [56]. Thus, a panel of immunostains including chromogranin, synaptophysin, calcitonin, CK7, CK20, TTF1, and CDX2 is helpful to determine the primary site for cutaneous metastases of a neuroendocrine tumor.

Focal positivity for neuroendocrine markers can be misleading in skin metastases as adenocarcinomas from many sites can show a degree of neuroendocrine differentiation [58]. A study of 356 adenocarcinomas chromogranin showed positivity in 41.5% of colon, 39.6% of gastric, 38.1% of prostate, 21.0% of breast, and 17.9% of pancreatic carcinomas [58]. Additionally, true neuroendocrine tumors of the breast are uncommon, but can involve the nipple areolar complex and have been confused with MCC [59]. Thus, one must maintain a broad differential diagnosis when evaluating tumors that show focal neuroendocrine differentiation.

20.3.3 Paraganglioma and Pheochromocytoma Metastases to the Skin

Paragangliomas and adrenal pheochromocytomas can metastasize to the skin and need to be differentiated from other neuroendocrine tumors [60, 61]. Unlike neuroendocrine carcinomas, paragangliomas and pheochromocytomas are negative for keratin, with the exception of those of cauda equina. A primary cutaneous paraganglioma has also been described [62]. In addition to neuroendocrine tumors, paraganglioma-like dermal melanocytic tumor mimics paraganglioma [63]. However, these melanocytic tumors are positive for S100, Melan-A, and HMB-45. The S100 positivity in paragangliomas is limited to sustentacular cells.

20.4 Conclusion

Merkel cell carcinomas are aggressive tumors occurring in the sun-damaged skin of elderly patients. These tumors must be differentiated from the metastases to the skin. A variety of tumors can show neuroendocrine differentiation, thus careful incorporation of clinical, histologic, and immunophenotypic data is necessary for appropriate diagnosis. The pathogenesis of many of these tumors remains unknown, but the recent discovery of Merkel cell polyomavirus DNA integrated in the tumor genome in a clonal pattern indicates that a virus may have an etiologic role in MCC. Although there remains much to be learned, this discovery has been a significant advance in the understanding of the pathogenesis of these cutaneous neuroendocrine tumors.

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Chapter 21
Neuroendocrine Carcinomas of the Thymus

Cesar A. Moran and Saul Suster

21.1 Introduction

Thymic neuroendocrine carcinomas are tumors of unusual occurrence. It has been estimated that they account for no more than 5% of all mediastinal tumors and that they also behave more aggressively in approximately 80% of the cases. Rosai and Higa are credited for the first description of these tumors in the thymic region after their description of 8 cases [1]. Rosai et al. are also credited for highlighting the association of these tumor with the multiple endocrine neoplasia (MEN) syndrome [2]. The authors stated that this association might prove to follow a more aggressive behavior than tumors not associated to this syndrome.

Since those initial descriptions of thymic neuroendocrine carcinomas (carcinoids), numerous other descriptions of similar tumors have followed, some emphasizing clinical aspects and others emphasizing histopathological aspects [3–13]. More recently, a larger study comprising 80 primary neuroendocrine carcinomas has been presented highlighting a new classification scheme when these tumors occur in the thymic region, as well as the diverse clinical conditions that may be associated with these tumors [14].

21.2 Clinical Aspects

Thymic neuroendocrine carcinomas are a group of tumors more commonly associated to the MEN, type I endocrinopathy, which, as some authors view, may alter the prognosis of these tumors. In that regard, it is possible that previous cases of thymomas associated with endocrinopathies such as Cushing's syndrome may in fact represent thymic neuroendocrine carcinomas as has been reported on other occasions. Nevertheless, thymic neuroendocrine carcinomas may also be associated with other conditions including polyarthropathy, proximal myopathy, and peripheral neuropathy, hyperparathyroidism, incomplete Sipple syndrome (MEN-II), ADH secretion, Eaton-Lambert syndrome, hypertrophic osteoarthropathy, secretion of ACTH , and secretion of parathormone, calcitonin, beta-lipoprotein, and serotonin. Some authors have estimated that about half of all neuroendocrine carcinomas in the thymus are functionally active or associated to MEN while about 30% are malignant on the basis of local invasion, metastasis, or both. Interestingly carcinoids have not been associated with myasthenia gravis, carcinoid syndrome, or hypogammaglobulinemia.

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21.3 Gross Features

The tumors may be well circumscribed and limited to the anterior mediastinum or may infiltrate the pleura, pericardium, and lung. At cut surface they may show a tan color with a homogeneous surface while other tumors may show areas of hemorrhage and/or necrosis. The size of these tumors may vary from 1 to more than 5 cm in diameter. In addition, cystic tumors have been reported [15].

21.4 Histopathological Features

Thymic neuroendocrine carcinomas (carcinoids) recapitulate similar features as those in other anatomic areas such as the lung or gastrointestinal tract. More recently, a more expanded view of the different histopathologic growth patterns that may be observed in these tumors has been presented [16–22].

These tumors are characterized at the low power view by a prominent nesting pattern and a homogeneous growth. The nests are separated by thin fibrocollagenous tissue while in other areas the growth pattern is that of ribbons of cells exhibiting similar cytological features (Figs. 21.1–21.6). The characteristic cytology is that of small or medium-sized cells with moderate amounts of pale eosinophilic cytoplasm, round to oval nuclei, and inconspicuous nucleoli. The tumors on occasions may show a prominent oncocytic differentiation in

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which the tumor cells appear a little larger than the conventional growth pattern. In this setting the cells show moderate amounts of prominent eosinophilic cytoplasm and the nuclei appear to be more prominent. However, the nucleoli are still inconspicuous. Thymic neuroendocrine carcinomas with prominent spindle cell features may also be seen. In these cases the cells adopt a fusiform shape mimicking a mesenchymal tumor. In some cases melanin pigment may be observed in any of the growth patterns and these tumors are regarded as pigmented neuroendocrine carcinomas (carcinoids). In very unusual circumstances the tumor may display a characteristic angiectatic growth pattern similar to that observed in vascular tumors. In these tumors, the presence of large ectatic areas filled with red cells may be confused with a vascular tumor. However, the presence of these ectatic areas is one of the typical cytological features of a neuroendocrine tumor. Also important to note is the presence of a tumor in which the neoplastic cells are embedded in an acellular eosinophilic amyloid-like stroma. Tumors showing this type of growth pattern may be confused with tumors of a different origin such as thyroid medullary carcinoma.

Two additional unusual variants that are important to recognize include the mucinous thymic neuroendocrine carcinoma (carcinoid) and tumors that share combined features of low and high-grade differentiation. In the former, the tumor cell population may be scant and embedded in large pools of mucin which may be confused with a primary mucinous carcinoma of lung origin while in the latter, the tumor shows alternating areas of conventional "carcinoid" admixed with other areas more in keeping with conventional "small cell carcinoma." It is important to keep these two histopathological growth patterns in mind, especially when limited mediastinoscopic biopsies are the only diagnostic material available. In this context, it is also important to mention that neuroendocrine carcinomas (carcinoids) may also be associated or admixed with other neoplasms such as thymic carcinoma or mesenchymal tumors.

21.5 Immunohistochemistry and Ultrastructure

We were able to analyze 40 cases of primary thymic neuroendocrine carcinomas using a panel of antibodies which included CAM 5.2 low molecular weight keratin, broad spectrum keratin cocktail, chromogranin, synaptophysin, and Leu-7. In our experience, all our cases showed strong positive reaction for CAM5.2 while broad-spectrum keratin was positive in approximately 88% of the cases studied. Of the neuroendocrine markers tested, chromogranin was seen positive in 75%, synaptophysin in 73%, and Leu-7 in 68%. In only 60% of the cases studied a dual staining with chromogranin and synaptophysin was observed. Interestingly, in our experience, p53 was seen only focally positive in less than 5% of the cases studied.

Ultrastructurally, the finding of neurosecretory granules in tumor cells is the most important feature. However, the presence of neurosecretory granules is more readily seen in better-differentiated neoplasms.

21.6 Classification

Although histologically speaking thymic neuroendocrine carcinomas are similar to those seen in other areas such as the lung, great care must be exercised in their classification as the prognosis for these tumors in the thymus is different from those in the lung or gastrointestinal tract. Thus, we have modified the approach and nomenclature of these tumors when they occur in the thymus following the notion already presented by others that these tumors represent a spectrum of differentiation. Nevertheless, it must be understood that the classification scheme takes into account not only the presence of necrosis, cellular atypia, and mitotic count but also the fact that in order to provide a more precise classification, a surgical resection of the mediastinal tumor must take place. The use of this classification based on mediastinoscopic biopsies may prove inaccurate. A more comprehensive review of the entire issue of the diagnosis of neuroendocrine carcinomas has been presented in order to address these specific issues [23]. Therefore, we recommend the following criteria for the diagnosis of neuroendocrine carcinomas.

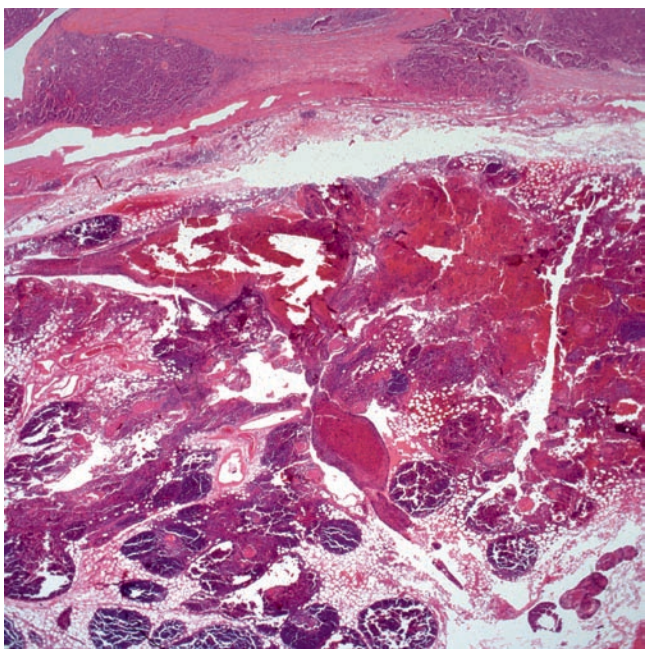

Fig. 21.1 Low power view of a thymic well-differentiated neuroendocrine carcinoma. Note the presence of thymic tissue in the periphery of the tumor

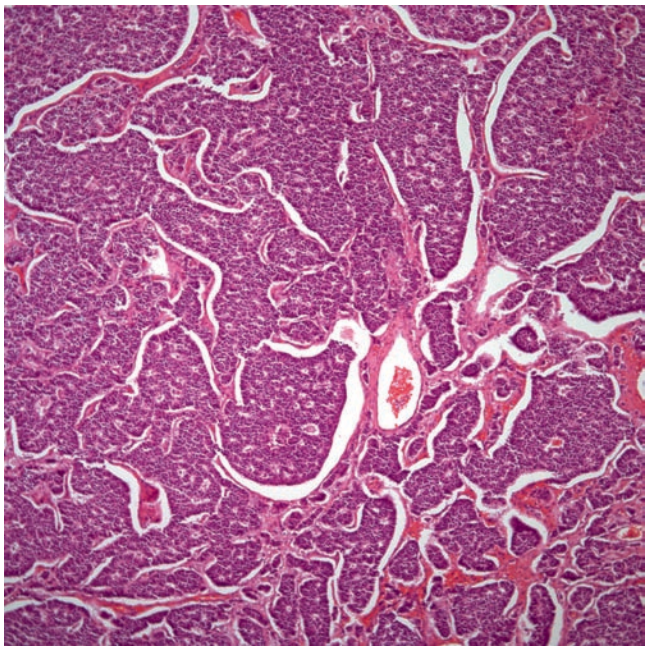

Fig. 21.2 Intermediate-grade magnification of a well-differentiated neuroendocrine carcinoma showing a homogenous cellular proliferation

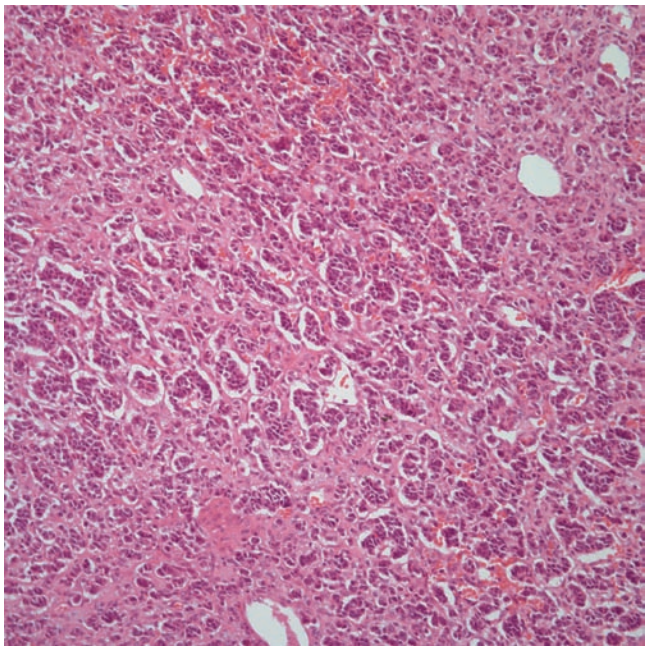

Fig. 21.4 Moderately differentiated neuroendocrine carcinoma showing less organized pattern of growth

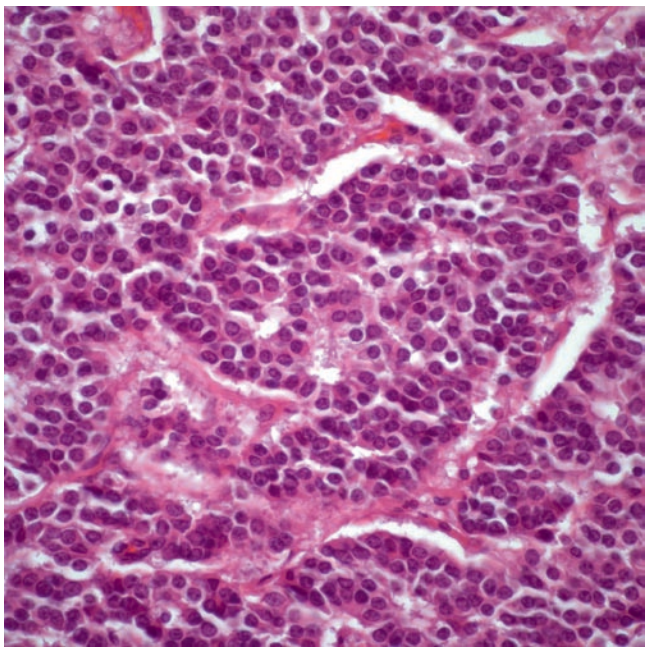

Fig. 21.3 High power magnification showing tumor cells without evidence of mitotic activity

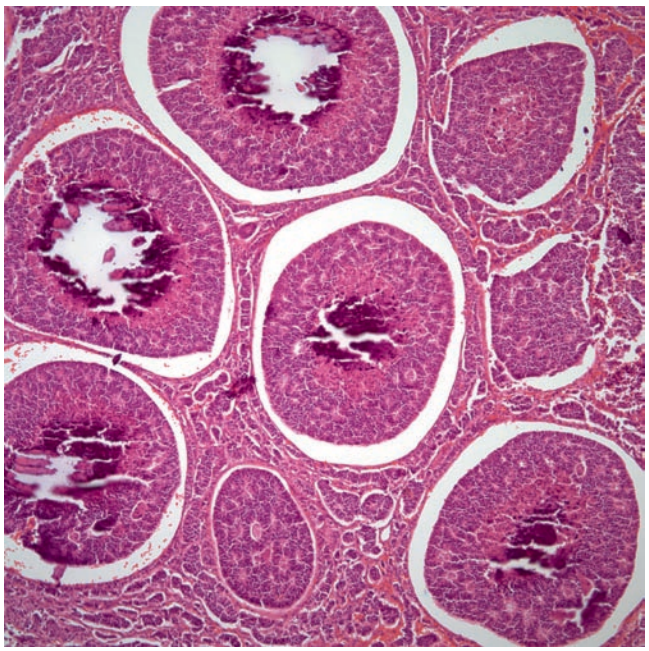

Fig. 21.5 Moderately differentiated neuroendocrine carcinoma showing the characteristic comedo-like necrosis

1. Well-differentiated (low-grade) neuroendocrine carcinoma (conventional carcinoid)
   - Mild cellular atypia
   - Fewer than 3 mitotic figures × 10 hpf
   - Small foci of comedonecrosis
2. Moderately differentiated (intermediate-grade) neuroendocrine carcinoma (atypical carcinoid):
   - Moderate cellular atypia
   - 3—9 mitotic figures × 10 hpf
   - More extensive foci of necrosis

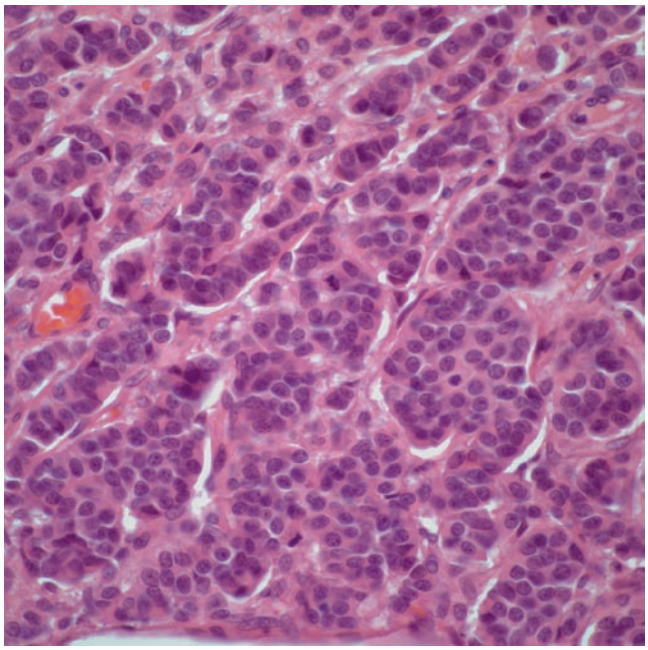

Fig. 21.6 High power magnification of a moderately differentiated neuroendocrine carcinoma showing two mitotic figures

3. Poorly differentiated (high-grade) neuroendocrine carcinoma (small cell carcinoma)
    - Severe or prominent cellular atypia
    - More than 10 mitotic figures × 10 hpf
    - Extensive areas of necrosis

It is important to note that some of these tumors may show overlap of features and mix histologies. Therefore, careful interpretation of the different histologic grades is necessary.

21.7 Differential Diagnosis

The most important considerations regarding primary neuroendocrine carcinomas of the thymus include metastatic neuroendocrine carcinoma from other sources such as the lung, mediastinal paraganglioma, and ectopic parathyroid adenoma. In cases of metastatic tumors from the lung, a precise interpretation of thoracic radiographs plays an important role in properly assessing the origin of the tumor. Paraganglioma and parathyroid adenomas pose a more difficult problem as both tumors are by definition neuroendocrine in nature [24, 25]. In paragangliomas, the histopathologic characteristic is that these tumors will show a similar growth pattern as neuroendocrine carcinomas. However, they are also characterized by the presence of large "megalic" cells with bizarre forms and shapes but very few mitotic figures if any. In addition, paragangliomas will display negative staining for keratin while neuroendocrine carcinomas show for the most part positive staining. In cases of parathyroid adenomas, the presence of prominent clear cells (chief cells) admixed with oncocytic cells may lead to the correct interpretation. In addition, the use of periodic acid-Schiff to determine the presence of glycogen and the use of immunohistochemical studies for parathyroid hormone will also be helpful in this setting.

21.8 Prognosis

On the basis of our experience, we consider that the prognosis is linked to the degree of differentiation of these tumors. In those tumors showing better differentiated features, it is expected that the survival rate is around 50% at 5 years, those showing moderately differentiated features 20% at 5 years, and those showing poorly differentiated features 0% at 5 years. Therefore, we consider that every attempt should be made to properly classify these tumors accordingly to the degree of differentiation. In this particular issue, we disagree with the current nomenclature proposed by the World Health Organization of classifying thymic neuroendocrine carcinomas into low and high grade tumors [26]. This approach is not supported by any of the current series of cases dealing with these particular tumors.

21.9 Analysis

It has been almost 100 years since the term "carcinoid" was introduced in the literature in order to distinguish a group of tumors in the small intestine that behave better than conventional carcinomas [27]. In time, the same type of tumor was also described in other anatomic areas and in some of them, time has proven that the behavior is not as innocuous as once was thought. Thus, we have proposed to abandon the term "carcinoid" for a more appropriate term, neuroendocrine carcinoma. It is hoped that by providing this "more meaningful" approach, more research can be done in terms of better therapeutic panels to improve the life expectancy of patients with these tumors. We also believe that the term neuroendocrine carcinoma with its different grades of differentiation denotes the spectrum of differentiation that these tumors may show when they occur in the thymic region.

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Chapter 22
Endocrine Placenta

Raymond W. Redline

Abbreviations

ADAM	a disintegrin and metalloproteinase
ANG-2	angiopoietin 2
AT-1	angiotensin receptor, type 1
cAMP	cyclic adenosine monophosphate
ChorioCA	choriocarcinoma
CEACAM-1	carcinoembryonic antigen-like cellular adhesion molecule
CRH	corticotropin-releasing hormone
CRH-BP	corticotropin-releasing hormone binding protein
cT	cytotrophoblast
DHEA	dehydroepiandrosterone
E2	estradiol
E3	estriol
EpT	epithelioid trophoblast
ETT	epithelioid trophoblastic tumor
EvT	endovascular trophoblast
hCG	human chorionic gonadotropin
hGH-V	human growth hormone-variant
hPL	human placental lactogen
HIF-1	hypoxia-induced factor 1
HSD	11-beta hydroxysteroid dehydrogenase
IGF	insulin-like growth factor
IGF-II/MBP receptor	insulin-like growth factor II/ mannose-binding protein receptor
IGF-BP	insulin-like growth factor binding protein
iT	intermediate trophoblast
LDL	low-density lipoprotein
P4	progesterone
PAI-1	plasminogen activator inhibitor 1
PAPP-A	pregnancy-associated plasma protein
PGDH	prostaglandin dehydrogenase
PGE2	prostaglandin E2
PlGF	placental growth factor
PP13	pregnancy protein 13
PPAR-γ	peroxisome proliferator activation receptor gamma
PSN	placental site nodule
PSTT	placental site trophoblastic tumor
ScT	syncytiotrophoblast
sENG	soluble endoglin
sflt-1	soluble vascular endothelial growth factor-receptor type 1
TGF-β	transforming growth factor beta
TNF-α	tumor necrosis factor-alpha
VEGF	vascular endothelial growth factor

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22.1 Introduction

The placenta differs from the specialized endocrine organs by virtue of its primary nonendocrine function and the wide variety of different hormones and receptors it expresses during the various stages of pregnancy. These hormones are primarily produced by trophoblast, the principal cell type of the placenta. Among the processes coordinated by placental trophoblast are uterine implantation, remodeling of the maternal circulation, maintenance of ovarian steroid production, formation of a shared vasculature with the fetus, regulation of maternal metabolism to maximize substrate delivery, transport of metabolites across the maternal–fetal interface, adaptation to intrauterine stress, protection from microorganisms and other teratogens, and triggering the signal cascade leading to labor and parturition.

Placental functions are compartmentalized. For the purposes of this chapter, I will separately consider endocrine physiology at three maternal–fetal interfaces within the placenta (Fig. 22.1a–c). The *implantation site* is composed of tissue-invasive intermediate trophoblast (iT), angioinvasive endovascular trophoblast (EvT), placental site giant cells, and maternal uterine tissues (decidua/endometrium

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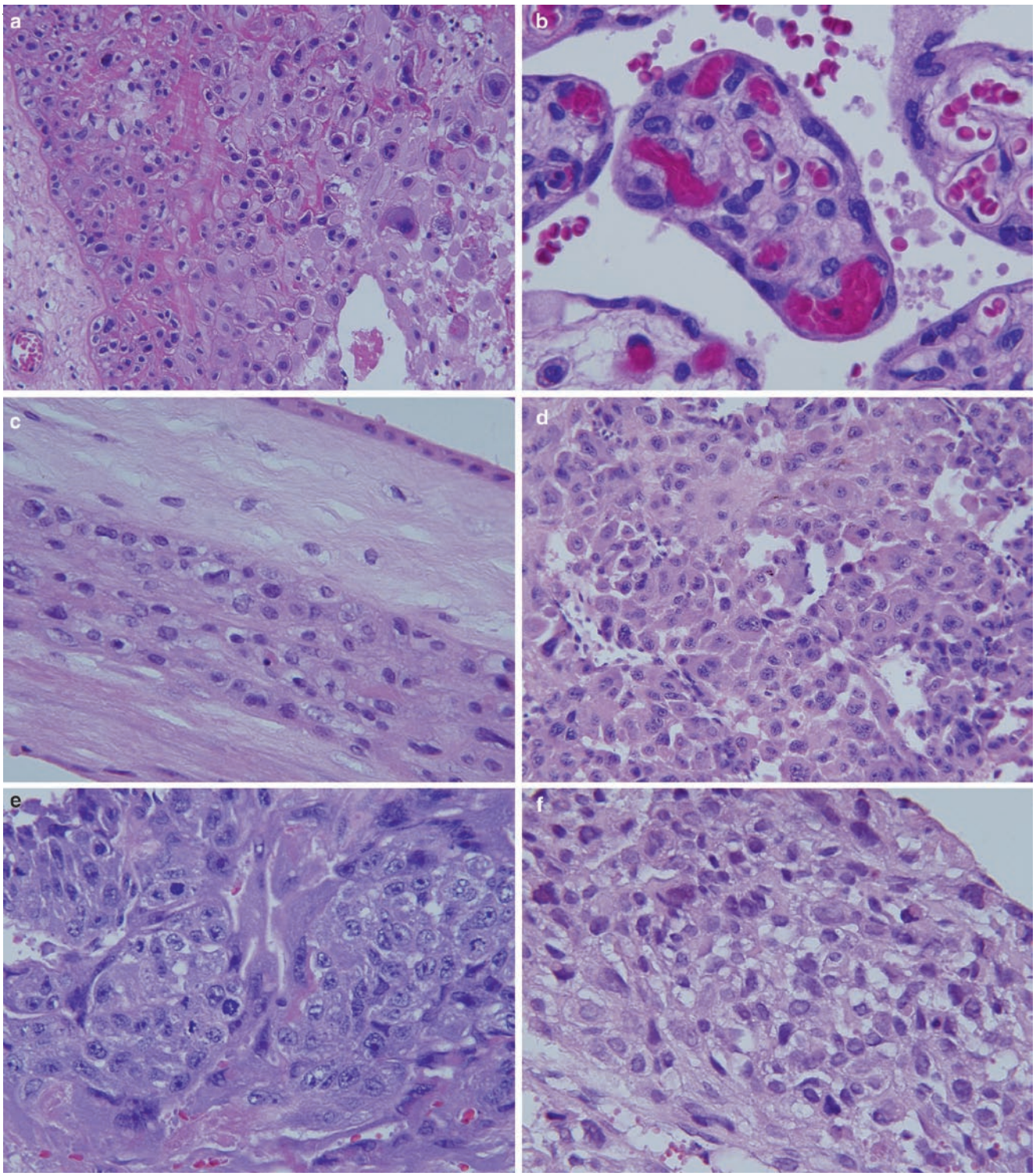

Fig. 22.1 Histology of the three maternal–fetal interfaces and tumors derived from them. (**a**) Implantation site: An anchoring villus is seen at the *far left*. In the *center left*, proximal intermediate trophoblast are embedded in a fibrinoid matrix rich in fibronectin. In the *center right*, more discohesive distal intermediate trophoblast infiltrate the maternal decidua. At the *far right*, a few placental site giant cells are seen (20× magnification). (**b**) Interhemal membrane: A single chorionic villus at the *center* is surrounded by a continuous monolayer of synctiotrophoblast separating fetal capillaries and villous connective tissue from surrounding maternal blood in the intervillous space. Villous cytotrophoblast stem cells are inconspicuous in late pregnancy and are not shown (60× magnification). (**c**) Extraplacental membanes: There are three distinct bands of tissue. At the *top* is the amnionic epithelium and its underlying connective tissue. At the *bottom* is the decidualized maternal endometrium. Separating these layers is the chorionc laevae, composed of a band of vacuolated epithelioid trophoblast (40× magnification). (**d**) Placental site trophoblastic tumor: the tumor is composed of sheets of partially cohesive tumor cells with dense eosinophilic cytoplasm resembling the distal intermediate trophoblast of the normal implantation infiltrate (20× magnification). (**e**) Choriocarcinoma: Clusters of atypical cytotrophoblast are surrounded by multinucleate syncytiotrophoblast similar to those surrounding the villi at the interhemal membrane (40× magnification). (**f**) Epithelioid trophoblastic tumor: Nodules of vacuolated tumor cells resembling the epithelioid trophoblast of the extraplacental membranes infiltrate the uterus (40× magnification)

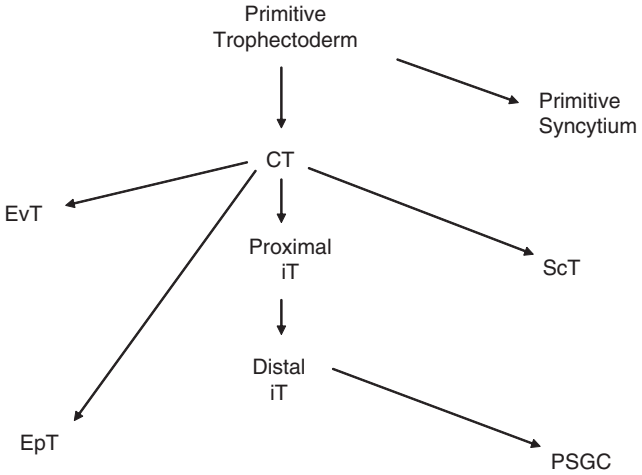

Fig. 22.2 Trophoblast differentiation pathway. Primitive trophectoderm covering the blastocyst differentiates to form a primitive syncytium that invades the endometrial stroma during early implantation. Cytotrophoblast stem cells (*cT*) derived from the trophectoderm give rise to three distinct lineages. Endovascular trophoblast (*EvT*) grows down the lumina of the spiral arterioles forming occlusive plugs that will be dissolved at 8–10 weeks' gestation. Epithelioid trophoblast (*EpT*) aggregates in the collapsed intervillous space of the membranes forming a continuous layer separating fetal amnion from maternal decidua. Proximal intermediate trophoblast (*Prox iT*) delaminates from the basement membrane of the anchoring villi in the maternal endometrium. After several rounds of proliferation, proximal intermediate trophoblast become invasive distal intermediate trophoblast (*Dist iT*) which infiltrate the uterine wall and remodel the spiral arterioles. Distal intermediate trophoblast eventually coalesce to form placental site giant cells (*PSGC*) at the maximum depth of implantation, usually in the superficial myometrium

and myometrium). It is the site where the placenta forms a firm attachment to the uterus and remodels maternal blood vessels ensuring that an appropriate volume of maternal blood enters the placenta at each developmental stage. The *interhemal membrane*, consisting of cytotrophoblast (cT) and syncytiotrophoblast (ScT), is the primary site of maternal–fetal transport and hence is heavily involved in the regulation of maternal availability and fetal access to nutrients. The *extraplacental membranes* are composed of fetal amnion and maternal decidua separated by a distinct chorionic layer of epithelioid trophoblast (EpT). Its primary functions are protection of the fetus and coordination of the timing and progression of labor and delivery. While for the purposes of discussion the types of trophoblast in each of these compartments are treated as distinct, it is important to emphasize that cT stem cells can generate all types of trophoblast at any location under stress. For example, culture of denuded first trimester villous explants will normally regenerate a covering of ScT. However, if cultures are supplemented with fibroblast growth factor-2 and heparin, intermediate and EpT are generated instead [1]. The relationship between the various trophoblast lineages is shown in Fig. 22.2. Expression profiles for the major classes of placental hormones are summarized for quick reference in Table 22.1.

Table 22.1 Expression of selected hormonal ligand and receptors in specific placental cell types

Hormone	cT	ScT	Prox iT	Dist iT	EvT	DSC	EpT
HCG	–	+	–	–	–	–	–
E2, E3, P4	–	+	–	–	–	–	–
HPL	–	+	+	+	+	–	–
HGH-V	–	+	+	+	+	–	–
PRL	–	–					
Leptin	–	+					
PPAR-γ	+	++	++	++	+		+
IGF-II	+	–	+	+		–	+
IGF-BP1-6	–	–				+	
PAPP-A	–	+	+	+			
Activin A			+	+	–		
Inhibin A	–	+ early	–	+	+	–	+
ENG/sENG	–	+	+	+			
VEGF-A	–	–	–	+	+	–	
PlGF	–	+	–	+	+	–	
ANG-2	–	+	–	+			
VEGF-R1/ sflt-1	–	+	+	+			
CRH	–	+	–	–	–	–	+
CRH-BP		+					+
HSD-1		–				+	+
HSD-2		+				+	
PGDH		+					+
Prorenin						+	

CRH corticotropin releasing hormone, *CRH-BP* corticotropin releasing hormone binding protein, *DSC* decidual stromal cell, *E2* estradiol, *E3* estriol, *EpT* epithelioid trophoblast, *EvT* endovascular trophoblast, *hCG* human chorionic gonadotropin, *hGH-V* human growth hormone-variant, *hPL* human placental lactogen, *HSD-11* beta-hydroxysteroid dehydrogenase, *IGF* insulin-like growth factor, *IGF-BP* insulin like growth factor binding protein, *iT* intermediate trophoblast, *P4* progesterone, *PAPP-A* pregnancy associated plasma protein, *PGDH* prostaglandin dehydrogenase, *PlGF* placental growth factor, *PPAR-gamma* peroxisome proliferator activation receptor gamma, *PRL* prolactin, *PSGC* placental site giant cell trophoblast, *sENG* soluble endoglin, *sflt-1* soluble vascular endothelial growth factor-receptor type 1, *TIMP-3* tissue inhibitor of metalloproteinase- 3, *vCT* villous cytotrophoblast, *VEGF-R1* vascular endothelial growth factor-receptor type 1

22.2 Implantation Site

22.2.1 Development and Structure

Anchoring villi in contact with the maternal endometrial stroma develop a proliferative population of cT that acquire migratory and tissue-invasive properties including expression of integrin receptors, metalloproteinases, and chemokine receptors and loss of cell junction components such as E-cadherin and gap junction proteins [2, 3]. This change in phenotype allows deep infiltration of the endometrium and superficial myometrium in normal pregnancy. Extravillous trophoblast can be separated into two groups: those closest to the anchoring villus that remain relatively cohesive [(proximal intermediate trophoblast (Prox iT)]

and those further from the anchoring villus that are more discohesive and invade the decidua [distal intermediate trophoblast (Dist iT)]. The proximal cells differentiate into fibronectin-secreting cells that form Nitabuch's layer of the basal plate in later pregnancy. The distal cells coalesce to form placental site giant cells at the maximum depth of implantation, usually in the superficial myometrium. The placental site giant cells strongly express plasminogen activator inhibitor 1 (PAI-1), which may serve as an autocrine mediator restricting further invasion [4]. Some of the Dist iTs adopt a stellate configuration as they surround and invade maternal arteries [5–7]. A third population of extravillous trophoblast known as EvT migrate down from the cT shell within the lumen of maternal arteries to form plugs that retard arterial blood flow into the fragile intervillous space for the first 8–10 weeks of gestation [8]. Later they invade the vessel wall and together with the stellate iT replace the vascular smooth muscle cells with a fibronectin-rich extracellular matrix substance. The resulting remodeled arteries are dilated and protected from vasospasm allowing the consistent flow of a large volume of maternal blood into the intervillous space during the period of maximum fetal growth in the late second and third trimesters. The uterine veins are not remodeled, a distinction which may relate to lack of ephrin ligands which are bound by trophoblast within the arteries [9].

22.2.2 *Endocrine Mediators*

Transforming growth factor beta (TGF-β) family members are associated with inhibition of extravillous trophoblast function at a variety of levels including downregulation of metalloproteinases and integrin receptors associated with invasion and upregulation of protease inhibitors [3, 10]. Low oxygen tension in the early implantation site has been implicated as the primary regulator leading to increased expression of hypoxia-induced factor 1 alpha (HIF-1α) and elevated levels of TGF-β3 [11]. The effects of TGF-β are accentuated by endoglin, a coreceptor in the TGF-β3 signaling pathway, which is also expressed at high levels during this time period [12]. In normal pregnancies, the tissue oxygen tension increases and endoglin levels decrease [13]. In abnormal pregnancies, endoglin expression persists, and high levels of a soluble endocrine form of endoglin are cleaved from the trophoblast surface and enter the maternal circulation [14]. Soluble endoglin (sENG) may interfere with TGF-β-mediated vascular remodeling in the uterus and also has systemic vascular effects that reduce uterine perfusion [13]. Other TGF-β family members expressed on extravillous trophoblast include activin, inhibin, and the activin-binding protein, follistatin. In vitro evidence suggests that, in contrast to TGF-β, activin promotes invasion by first trimester extravillous trophoblast [15, 16]. Expression and invasion are both inhibited by follistatin. Inhibin had no activity in this system. Whether endoglin or sENG modulate local activin receptor function is unknown.

Members of the vascular endothelial growth factor (VEGF) and angiopoietin (ANG) families and their receptors are ubiquitiously expressed in the implantation site [17, 18]. VEGF-A, placental growth factor (PlGF), and ANG-2 are all upregulated in Dist iT. VEGF receptors 1, 2, and 3 are also expressed on extravillous trophoblast, whereas ANG receptors are confined to the uterine vasculature. Nonheparin binding forms of VEGF-A promote trophoblast proliferation in vitro, and functional blockade of VEGF-R1 leads to decreased invasion, increased apoptosis, and the downregulation of adhesion receptors implicated in tissue invasion and vascular remodeling [17, 19]. PlGF is known to play a critical role in postischemic and tumor angiogenesis, situations that may model some aspects of maternal vascular remodeling in the implantation site. ANG-2 can promote angiogenesis, and blockade of its receptor (Tie 2) in vitro has been shown to result in apoptosis of uterine microvascular endothelial cells [18]. Interestingly, decreased oxygen tension in pathological pregnancies also leads to the HIF-1α-dependent expression of a soluble endocrine form of VEGF-R1 known as soluble vascular endothelial growth factor-receptor type 1 (sflt-1) [20]. This mediator, like sENG, can act as a potent inhibitor of both local trophoblast and systemic endothelial function leading to defective placentation and decreased uterine perfusion [21, 22].

Insulin-like growth factor (IGF)-II is also upregulated in parallel with the invasive phenotype in Dist iT [23]. Autocrine signaling via insulin-like growth factor II/mannose-binding protein (IGF-II/MBP) receptors results in proliferation, decreased apoptosis, and increased expression of metalloproteinases [24]. IGF-II/MBP receptor expression on iT is upregulated by human chorionic gonadotropin (hCG), which has been implicated in early trophoblast invasion by several investigators [25, 26]. Conversion of proIGF-II to IGF-II is catalyzed by PC4, which is decreased in fetal growth-restricted pregnancies [27]. IGF-II activity is inhibited by the IGF-binding proteins (IGF-BP1-6), all of which are expressed in the decidua [23]. Bioavailablility of IGF-II depends on the expression of IGF-BP proteases such as pregnancy-associated plasma protein (PAPP-A), PAPP-A-related protein, a disintegrin and metalloproteinase (ADAM) 12, and IGF-BP3. PAPP-A, which is expressed in trophoblast and induced in decidual cells by IGF-II, cleaves IGF-BP4 and is particularly important for IGF-II function [28, 29]. A trophoblast-derived protein, pro-major basic protein, functions as an inhibitory binding protein for PAPP-A [30]. Decreased expression of PAPP-A is strongly associated with IUGR in both animal and human pregnancies [28]. The role of the most abundantly expressed binding protein, IGF-BP1, is more ambiguous. It

primarily regulates IGF-I, not IGF-II, function, and is relatively inactive in the dephosphorylated states that predominate in pregnancy [23, 31]. Other regulatory effects of IGF-BP1 have been proposed such as promotion of decidualization, inhibition of vascular remodeling, and direct regulation of binding to α5 β1 integrins on iT via an RGD-binding sequence [23, 32, 33].

The decidua expresses high levels of prorenin, renin, angiotensinogen, angiotensin receptors, and angiotensin-converting enzyme; trophoblast expresses high levels of AT-1 receptor [34, 35]. Recently, it has been shown that AT-1-dependent binding of angiotensinogen II by trophoblast results in the release of high levels of sflt-1 and PAI-1, which may inhibit trophoblast invasion and lead to maternal underperfusion [36]. AT-1 receptor activating autoantibodies, which have been detected in the serum of preeclamptic patients, have similar effects and together with dysregulated expression of other placental specific renin-angiotensin components may play a critical role in the systemic effects of preeclampsia [34].

Other less well-established regulators of trophoblast invasion include the following. Leptin and human growth hormone-variant (hGH-V, see below) have been associated with increased trophoblast invasiveness [37, 38]. Peroxisome proliferator activation receptor gamma (PPAR-γ) is ubiquitously expressed in trophoblast, and activators of its function such as oxidized low-density lipoprotein (LDL) have been reported to inhibit extravillous trophoblast invasion via interaction with surface scavenger receptors [39, 40]. Potential target genes negatively regulated by activated PPAR-γ include hGH-V and PAPP-A. Oxidized LDL may also interact with nuclear liver receptor X to restrict invasion. Some evidence suggests that corticotropin-releasing hormone (CRH) (discussed below) may inhibit trophoblast invasion by downregulating the expression of carcinoembryonic antigen-like cellular adhesion molecule (CEACAM-1) [41]. CEACAM-1 is a carcinoembryonic family member that colocalizes with osteopontin and interacts with β3 integrins at the iT invasion front [42]. Estrogen has recently been shown to decrease trophoblast-dependent vascular remodeling in an in vivo primate pregnancy model [43]. This effect was associated with an increase in uterine venous sflt-1 and decreased VEGF mRNA in Prox iT. Finally, some evidence suggests that heme oxygenase-1 expression in Dist iT may promote vascular remodeling by a variety of mechanisms including downregulation of sflt-1 and sENG [44–46].

22.2.3 Clinical Aspects

The most important clinical complication associated with decreased invasion of the uterus and inadequate remodeling of maternal arteries is preeclampsia [47]. Preeclampsia is a systemic maternal hypertensive illness affecting approximately 3–5% of pregnant women. It is a leading cause of both maternal and fetal morbidity and mortality. These complications relate to severe maternal endothelial dysfunction, premature placental separation, and underperfusion of the placenta. Predisposing factors include primiparity, maternal obesity, underlying maternal vascular disease, excessive volume of placental trophoblast, assisted reproductive technologies especially when associated with nonmaternal/paternal donors, and maternal thrombophilia. A strong genetic predisposition independent of these factors is also suspected. The current thinking regarding pathogenesis is that early uterine hypoxia leads to defective placentation and the release of circulating mediators such as sflt-1 and sENG, as described above. Recurrence is most frequent with increased severity, presentation before term, underlying medical risk factors, and placental findings associated with severe maternal underperfusion. Both mothers and infants from affected pregnancies show an increased risk of later cardiovascular disease that may be genetic or programmed in utero (discussed later).

There is considerable interest in identifying maternal screening tests that might predict preeclampsia during early pregnancy. Identified women could then be treated (aspirin +/- heparin, calcium), followed closely (serial ultrasounds and pulsed flow Doppler studies), and delivered promptly before the onset of life-threatening maternal or fetal disease [47]. Initial reports of a specific association of sflt-1 levels with later risk of preeclampsia were rapidly followed by demonstration that slflt-1 levels are elevated in mothers with known risk factors such as primiparity, pregnancy at high altitude, and pregestational diabetics [20, 48, 49]. Levels in smokers, who are protected from disease, are decreased [50]. Various modifications such as determining sflt-1/PlGF ratio, the change in serum sflt-1 between 11–13 and 17–20 weeks, and combining sflt-1 with sENG have improved detection [48, 51, 52]. One of these reports found sflt-1 and sENG to be most useful by adding specificity to the more sensitive abnormal pulsed flow Doppler test (100% sensitivity/93% specificity for early onset preeclampsia) [52]. Other potential markers include decreased pregnancy protein 13 (PP13), PAPP-A, sex hormone-binding globulin, and adiponectin and increased Activin A, P-Selectin, and Inhibin A [53]. Sex hormone binding globulin and adiponectin are surrogates for insulin resistance, which is strongly associated with PET. PP13 is a cell surface protein in the galectin family that shows paradoxical kinetics in preeclamptic patients being underexpressed during the initial preclinical stages of abnormal placentation and overexpressed later in pregnancy with shedding of degenerative ScT microparticles during the symptomatic phase of disease [54]. Activin A and PP13 seem to be the most promising markers, the latter showing a

90% detection rate with 6% false positives when combined with pulsed flow Doppler testing [55, 56].

22.2.4 Neoplasia

Placental site trophoblastic tumors (PSTTs) are rare neoplasms composed of cells with phenotypic features of iT (Table 22.2) [57]. The tumors usually present as a discrete mass within the uterus, but are occasionally diffusely infiltrative. Microscopically, they are composed of semicohesive sheets of predominantly mononuclear cells with occasional binucleation (Fig. 22.1d). Cytoplasm is eosinophilic and the nuclei are hyperchromatic with prominent nucleoli. PSTTs usually follow term deliveries (95% of cases), often years after the preceding pregnancy. Occasional cases may be related to previous molar pregnancy, but the relationship is not as strong as for choriocarcinoma (ChorioCA) (see below). While generally indolent, 15–20% of tumors behave in a malignant fashion with local invasion and/or distant metastasis. Features predictive of malignant behavior include a mitotic rate greater than 5/10 high power fields, prominent necrosis, and less cytoplasmic eosinophilia. Although tumor cells are strongly positive for human placental lactogen (hPL), this is a poor clinical tumor marker as serum levels are not reliable. Occasional cells are weakly positive for hCG and serum hCG titers are usually less than 250 IUL. Some recent data suggests that an elevated percentage of serum-free β-subunit relative to total hCG may be a specific marker for PSTT, but this has been challenged and would be of limited clinical utility [58]. The precursor lesion for this tumor is unknown. Exaggerated implantation site has been proposed, but there is little solid evidence that this is a distinct entity. Placental site nodules (PSNs) are a better candidate, but their staining pattern is more consistent with another neoplasm, epithelioid trophoblastic tumor (ETT), which is discussed below. Biologically, virtually all PSTTs are 46,XX with an active paternal X chromosome [59], suggesting a pathogenic role for paternally expressed X-linked genes.

22.3 Interhemal Membrane

22.3.1 Development and Structure

Fetal connective tissue derived from the extraembryonic endoderm lining the inner surface of the cavity grows out into the primitive trophoblast syncytium of the implanting blastocyst. At the same time, lacunar spaces form within the syncytium and are filled by maternal blood from endometrial vessels that are eroded by the trophoblast. Fetal capillaries form in the connective tissue under the influence of growth factors from adjacent trophoblast and connect with larger vessels forming in the body stalk (later umbilical cord). The now vascularized fetal connective tissue expands and undergoes branching morphogenesis driving the formation of the villous trees. Expansion of the villous trees necessitates replacement of the primitive syncytium by a self-renewing villous trophoblastic epithelium that can cover, protect, and provide nutrients to the fetal tissues. In primates, this epithelium is composed of two cell types, cT stem cells and differentiated ScT. cT divides in an asymmetric manner generating a daughter stem cell and a more metabolically active cell that fuses with the existing ScT. Interestingly, a human endogenous retroviral protein, syncytin 1, appears to play a critical role in this fusion process [60]. Other important factors include ADAM proteins that are involved in the formation of multinucleate cells in other parts of the body (osteoclasts, skeletal muscle fibers, and fertilized eggs) and (pro)caspase 8 [61, 62]. The process of syncytialization continuously exposes phospholipids that can trigger maternal coagulation at the interface with the intervillous space [60]. These phospholipids are bound by Annexin 5 proteins, which mask their negative charge [63]. Individual "trophoblast proliferator units" composed of one cT with approximately ten ScT daughter nuclei are maintained throughout the remainder of the pregnancy [64]. Cell turnover occurs via the formation of syncytial knots composed of apoptotic ScT nuclei. These knots are shed into the maternal circulation to be replaced by newly created cells. The rate of syncytiotroblast fusion is decreased

Table 22.2 Use of hormonal markers and selected other ancillary diagnostic aids to diagnose gestational trophoblastic neoplasms and their precursor lesions

Lesion	hCG	hPL	Inhibin A	p63	p57/KIP2	Ploidy = 3 N
PHM	+ (ScT/iT)	+ (ScT/iT)	+ (ScT/iT)	+ (cT/epT)	+ (cT/iT/epT)	+
CHM	+ (ScT/iT)	+ (ScT/iT)	+ (ScT/iT)	+ (cT/epT)	− (cT)/+ (iT/epT)	−
ChorioCA	++ (ScT)	+ (ScT)	+ (ScT only)	+ (cT)	+ (cT)	−
PSN	+/−	+/−	+	+	+	−
ETT	+/−	+/−	+/−	++	+	−
PSTT	+/−	++	++	−	+	−

ChorioCA choriocarcinoma, *CHM* complete hydatidiform mole, *EpT* epithelioid trophoblast, *ETT* epithelioid trophoblastic tumor, *hCG* human chorionic gonadotropin, *hPL* human placental lactogen, *iT* intermediate trophoblast, *PHM* partial hydatidiform mole, *PSN* placental site nodule, *PSTT* placental site trophoblastic tumor, *ScT* syncytiotrophoblast

and the formation of syncytial knots is increased in response to underperfusion, hypoxia, and oxidative stress [65, 66]. When these stresses become severe, syncytiotroblast undergoes aponecrosis triggering villous agglutination and deposition of fibrin in the intervillous space [67].

22.3.2 Endocrine Mediators

22.3.2.1 Regulation of Maternal Homeostasis

hCG is one of the first proteins to be produced following syncytialization [68]. Elevation of intracellular cyclic adenosine monophosphate (cAMP) and another human retroviral protein, human endogenous retroviral protein hERV-2, are among the factors that are involved in expression [60]. The major role of hCG, by virtue of its homology to pituitary luteinizing hormone, is to sustain the steroidogenic function of the corpus luteum until the mass of trophoblast is sufficient to replace it as a source of estrogen and progesterone (P4). The hCG synthesized during very early pregnancy is more heavily glycosylated and may have an independent role in promoting trophoblast invasion through mechanisms described in "Development and Structure." HCG has also been shown to act in an autocrine fashion to promote cT to ScT differentiation [69].

Three pregnancy-specific hormones encoded in the hGH cluster on chromosome 17q22-24, human sommatomammotropins A and B (also known as the hPL), and hGH-V are expressed by trophoblast during pregnancy [70]. The two hPLs have identical amino acid sequences and together constitute fully 10–20% of all placental mRNA at term. Expression is constitutive and their primary role appears to be antagonism of insulin and activation of hormone-sensitive lipase resulting in an increased delivery of glucose, amino acids, and free fatty acids to the placenta. The primary role of hGH-V during pregnancy is induction of maternal IGF-I and its plasma-binding protein IGF-BP3. Downregulation of pituitary hGH-N, which has greater lactogenic activity, in the second trimester is coordinated with increased placental hGH-V expression and serum levels of hGH-V are closely correlated with maternal IGF-I levels and fetal growth until 28 weeks' gestation [71]. Expression of hGH-V is itself downregulated in the third trimester, but is maintained at higher levels in diabetic pregnancies [72]. This may in part be related to its upregulation by glucose, but could also reflect the delayed maturation and continuing growth of distal villi seen with some diabetic pregnancies.

The female sex steroids (P4 and the estrogenic hormones: estrone, estradiol (E2), estriol (E3), estetrol, and the catechol-estrogens) are initially produced in the corpus luteum during early pregnancy. They have protean functions including preparing the endometrium for implantation, enhancing uterine blood supply, inhibiting myometrial contractility, and promoting lactation [73–76]. The switch to primary placental production occurs at about 11 weeks. P4 synthesis depends on the uptake of cholesterol esters via trophoblast LDL receptors, and placental perfusion is the rate-limiting step. Estrogen upregulates the expression of LDL receptors and all of the major steroidogenic enzymes. However, while estrogen blockade decreases P4 synthesis, increased levels of exogenous estrogen do not further increase P4 production. Primate placentas lack enzymes required for de novo estrogen synthesis, but do express CYP19 aromatase allowing conversion of C19 precursor steroids to estrogens [77]. The major precursor for placental estrogen production is dehydroepiandrosterone (DHEA), which is supplied by both the mother and fetus [78, 79]. Fetal adrenal DHEA sulfate becomes the major substrate for placental estrogen synthesis after 20 weeks. The majority of fetal adrenal DHEA sulfate is 16-hydroxylated in the liver resulting in placental conversion to E3, a weak estrogenic hormone, which accounts for 90% of total placental estrogen production. Because of its dependence on maternal supply, placental transfer, fetal metabolism, and placental aromatization, maternal E3 levels have been utilized clinically as a general measure of fetoplacental function and in prenatal screening tests for both Down syndrome and increased risk of premature delivery.

22.3.2.2 Regulation of Fetal Homeostasis

ScT expresses receptors for hormones such as insulin, estrogen, glucocorticoids, leptin, and adiponectin, all of which affect metabolic functions in other tissues and some of which have been associated with changes in placental structure in experimental animal models [80]. Insulin receptors in early pregnancy are primarily localized on the ScT microvillous border, but later are concentrated on fetal endothelial cells where they may cooperate with angiogenic growth factors such as fibroblast growth factor and VEGF produced by trophoblast and villous macrophages to promote angiogenesis and new villous growth [81]. Glucose and amino acids are transferred across the interhemal barrier to the fetus by specific transporters expressed in ScT and fetal endothelium. Mice specifically deficient for a placenta-specific IGF-II transcript have very small placentas with an overall increase in diffusion distance, but have increased numbers of Glut 3 glucose and system A amino acid transporters per gram of tissue suggesting compensatory upregulation [80]. Varying changes in Glut 1 and Glut 3 transporter levels in more complex animal pregnancy models of hypoxia, maternal under perfusion, diabetes, and altered nutrition have also been reported but are difficult to interpret. Less is known about regulation of transporters in the human placenta. Increased maternal glucose upregulates and glucocorticoids downregulate placental Glut 1

[81]. AT-1 receptor agonists have been shown to downregulate the system A amino acid transporter [34].

A second type of endocrine regulation at the interhemal membrane protects the fetus from potentially harmful circulating maternal hormones. Current interest has centered on the enzyme 11-β hydroxysteroid dehydrogenase type II (HSD-2) [78]. This enzyme converts cortisol into corticosterone in the kidney, preventing occupation of the nonselective aldosterone receptor. Its expression in ScT is believed to protect the fetus from high maternal levels of cortisol that have the potential to cause reduced growth, premature tissue maturation, premature labor, and a predisposition to later cardiovascular disease (see below). HSD2 levels in trophoblast are decreased by undernutrition, stress-related cytokines, hypoxia, shear stress, nitrous oxide, and preeclampsia [82, 83]. They are upregulated by increased intracellular cAMP, elevated oxygen tension, and estrogen (baboon model) [77]. Families with loss of function mutations in HSD2 have a high rate of stillbirth [78]. Other potentially protective ScT enzymes include deiodinase (thyroid hormone) and CYP19 aromatase (androgens).

22.3.3 *Clinical Aspects*

Maternal serum screening for Down syndrome (Trisomy 21) relies on measurements of several hormones released into the maternal circulation at the interhemal membrane [84]. The biological basis for the abnormal hormonal levels in Down syndrome remains unclear but one factor may be impaired cT to ScT differentiation due to elevated levels of zinc-copper superoxide dismutase, an enzyme expressed on chromosome 21 [85]. The initial goal of screening was to find a combination of placental specific factors by empirical testing that would improve the 30% baseline sensitivity for detecting Down syndrome achieved by performing karyotypes based on advanced maternal age alone. The initial combination of decreased hCG, decreased E3, and increased α fetoprotein (nonhormonal) used at 10–15 weeks has now been supplemented by testing for increased dimeric inhibin A. An abnormal result for this combined "quad test" provides 80% detection with a 5% false positive rate. Another strategy performed at an earlier gestational age (11–13 weeks) utilizes decreased serum levels of PAPP-A, increased urinary levels of hCG free β subunit, and measurement of increased nuchal soft issue ("nuchal translucency") by ultrasound to give an 85% detection rate with 5% false positives. Finally, a combined early/late strategy of PAPP-A plus nuchal translucency at 11–13 weeks followed by quad test at 15 weeks provides the best overall performance with an 85% detection rate and 1% false positives. Other markers under study include increased urinary hyperglycosylated hCG and increased serum ADAM-12 [86, 87].

The fetal origins of adult disease hypothesis was proposed by Barker in 1993 based on the observation that low birth weight infants had a high incidence of later cardiovascular disease and early mortality [88]. Subsequent observations have found similar associations in large for gestational age infants [89]. These adverse long-term outcomes, particularly hypertension, are primarily seen after an intermediate stage of childhood obesity, either due to overexuberant catch up growth in low birth weight infants or continuing accumulation of adipose tissue in large for gestational age infants [90, 91]. The spectrum of adult diseases implicated in the fetal origins hypothesis has been expanded to include other components of the metabolic syndrome (e.g., atherosclerosis, obesity, insulin resistance, hyperlipidemia), chronic lung disease, and hypercoagulability [92]. An ongoing debate regarding the underlying pathogenesis centers around the relative role of predisposing genotypes inherited from mothers who have pregnancy complications versus the so-called thrifty phenotype in which in utero stress "programs" metabolic responses that may be useful for coping with later extrauterine deprivation [93]. If the anticipated extrauterine deprivation does not happen, overcompensation with obesity, hypertension, and other components of the metabolic syndrome may occur. Theoretically, the placenta could be an innocent bystander in this process. However, Barker also found that increased placental weight, irrespective of fetal weight, predicted similar outcomes [88]. Other observations such as morphological changes seen in some placentas from diabetic and hypertensive mothers, decreased ScT HDS-2 expression, and alterations in placentally derived hormones such as leptin raise the possibility that primary or secondary placental abnormalities might contribute to the adverse long-term outcomes found in these individuals [94].

22.3.4 *Neoplasia*

ChorioCA is a highly malignant tumor derived from villous trophoblast. The tumor has two components: clusters of atypical mononuclear villous cT and a wreath-like surrounding arcade of multinucleate ScT (Fig. 22.1e) [57]. The mononuclear cells have clear cytoplasm and large round central nuclei with chromatin clearing and a prominent nucleolus. The ScTs have purple cytoplasm and marked nuclear hyperchromasia. Large areas of necrosis and hemorrhage often accompany the tumor. ScT, but not cT, strongly expresses hCG, and levels of hCG are markedly elevated in the maternal serum. At least half of ChorioCAs arise from precursor lesions known as molar pregnancies, which have an overrepresentation of paternally derived chromosomes (androgenic lineage). The remainder follow apparently normal early and late pregnancies. The recent description of confined placental

mosaicism for androgenic lineages in otherwise normal term placentas (placental mesenchymal dysplasia) raises the possibility that an androgenic lineage is the common risk factor for most of these tumors. ChorioCAs are highly aggressive neoplasms with frequent metastases and were almost uniformly fatal in the prechemotherapy era. Currently, with combined chemotherapy and effective hCG tumor monitoring, they are curable in over 95% of cases. Adverse prognostic features include older maternal age, tumor size >5 cm, metastases to brain, liver, or GI tract, and recurrence after chemotherapy.

While ChorioCAs are rare, molar pregnancies are much more common especially among East Asians and Amerindians. Complete hydatidiform moles develop from totally androgenic conceptuses lacking female chromosomes. The resulting overrepresentation of growth-promoting paternal gene products together with the absence of regulatory maternal factors leads to failure of fetal development and trophoblast hyperplasia. Late in the course of complete moles, as with other anembryonic pregnancies, there is uniform swelling of the villi. However, early curettage of anembryonic pregnancies has made the pathologic diagnosis of complete mole more difficult due to lack of hydropic degeneration and relatively subtle trophoblastic proliferation [95]. Histologic clues such as hypercellular myxoid villous stroma, cauliflower-like villous growth pattern, and atypia of implantation site trophoblast are helpful features for diagnosis. Lack of immunostaining for p57/KIP2, a paternally imprinted gene not expressed in the villous trophoblast of androgenic pregnancies, is a useful ancillar test in problematic cases (Table 22.2). Partial hydatiform moles have two sets of paternal and one set of maternal chromosomes (diandric triploidy). They are morphologically dissimilar to complete moles in that they have two distinct populations of villi (one small and fibrotic, the other large and hydropic), irregular villous contours, trophoblast inclusions in the villous stroma, and a more normal gestational sac (fetus, umbilical cord, amnion, and fetal vessels). The degree of trophoblast hyperplasia, level of serum hCG, and risk of developing ChorioCA are all less than with complete mole, as might be predicted given their incompletely androgenic chromosomal status. Nevertheless, persistence of hCG titers following evacuation occurs in 0.5–20% of partial moles and occasional cases progress to ChorioCA. Immunostaining with p57KIP2 is not helpful in these cases, but fluorescence in situ hybridization or flow cytometry can be performed to confirm triploid status in difficult cases. The clinical approach to all types of molar pregnancy is the same. Serial serum hCG titers are followed for 1 year after evacuation. Failure of the titers to decrease appropriately is followed by single agent chemotherapy. Continuing persistence of elevated titers prompts diagnostic curettage, metastatic workup, and multiagent chemotherapy.

22.4 Extraplacental Membranes

22.4.1 Development and Structure

Formation of the placental membranes is easily understood. The implanting blastocyst is initially completely surrounded by chorionic villi. However, only the implantation site receives an adequate blood supply from the spiral arteries. In the remaining portions of the gestational sac, the intervillous space collapses, and the villi atrophy. cT stem cells in the collapsed intervillous space, freed from the regulatory influences of fetal and maternal perfusion, differentiate along two extravillous pathways. The iT pathway predominating in the implantation site was discussed previously. The second extravillous lineage, variably termed epithelioid, transitional, vaculolated, or chorion laevae extravillous trophoblast, has only recently been characterized. It is defined by coexpression of the tumor suppressor gene p63 and inhibin A and can be distinguished from iT by the absence of hPL [96–98]. These cells are a major component of the membranes, but can also be found in lesser numbers in the intervillous space and implantation site. Their proposed function is best understood in terms of their location. They form a continuous cell layer between the fetal amnion and the maternal endometrium and myometrium. As will be described in more detail below, they are believed to degrade prostaglandins formed in the amnion preventing premature activation of the labor cascade in the underlying uterus. Another possible role may be preventing the spread of microbes and immune effector cells from the mother into fetal tissues.

22.4.2 Endocrine Mediators

Placental CRH and corticosteroids from the mother and fetus are key mediators in the physiological processes controlling maturation of the fetal hypothalamic pituitary adrenal axis, activation of the myometrium, and the timing of parturition. CRH is expressed in the chorion laevae trophoblast and ScT of anthropoid, but not lesser apes [99]. CRH secretion levels per gram of placental tissue increase with advancing gestation, but activity is inhibited by placentally derived CRH binding protein (CRH-BP). Decline in CRH-BP levels with a corresponding increase in bioactive CRH immediately precedes labor in these species. Unlike many trophoblast hormones, CRH enters both the fetal and maternal circulations and in the former is critically important for the slow maturation of fetal adrenal adrenocortical trophic hormone and corticosteroid responses. In contrast to feedback inhibition in the hypothalamus, placental CRH

is actually upregulated by corticosteroids, so the maturation of the fetal adrenal and increased fetal cortisol further increases placental CRH production by a feedforward mechanism [100, 101]. Chronic hyoxia accelerates maturation of the fetal hypothalamic pituitary adrenal axis leading to premature upregulation of placental CRH [102]. Activation of toll-like receptor-4 receptors by LPS also activates placental CRH [103], whereas P4 inhibits it [99]. Placental actions of CRH include upregulation of EP1 prostaglandin receptors on amnionic epithelium and prostaglandin dehydrogenase (PGDH) in the chorion laevae [104]. Effects on the uterus are more complex. Signaling through CRH-R1 receptors promotes myometrial relaxation, while signaling through the CRH-R2 receptor contributes to uterine contractions [99].

Prostaglandin E2 (PGE2) is constitutively produced by mesenchymal cells in the amnion and upregulated in amnionic epithelium by cortisol and CRH [105]. A further layer of regulation is added by upregulation of the enzyme HSD-1 by prostaglandins in the placental membranes near term [78, 106]. This enzyme catalyzes the reaction of cortisone to cortisol (opposite of HSD-2, discussed above), and therefore increases the amount of bioactive cortisol and CRH in the membranes further amplifying production of prostaglandins. Access of these labor-inducing prostaglandins to the underlying myometrium is regulated by degradation in the intervening chorion laevae trophoblast. The degradative enzyme, PGDH, is upregulated by CRH and P4 and is downregulated by tumor necrosis factor-alpha (TNF-α), glucocorticoids, and increased levels of intracellular calcium [99, 104, 107, 108]. Downregulation in combination with upregulation of prostaglandin synthetic enzymes in the amnion, chorion, and myometrium results in increased levels of bioactive prostaglandins that together with CRH and oxytocin result in myometrial contractions.

The myometrium goes through four sequential states over the course of pregnancy and the puerperium [79]. In the quiescent phase, the effects of P4, prostacyclin, relaxin, parathyroid related protein, and nitric oxide predominate leading to myocyte relaxation. The relaxant effects of P4 may be mediated through nonnuclear P4 receptors such as progesterone membrane component-1 and -2 [109]. The second activation phase is triggered by placental estrogens, which are increased secondary to the placental CRH-mediated stimulation of fetal adrenal precursor synthesis in late pregnancy. Increased estrogen levels also lead to increases in gap junction proteins, ion channels, and receptors for uterotonic agents such as prostaglandings and oxytocin. The third phase, labor, is associated with increased levels of CRH, prostaglandins, and oxytocin. The final stage of uterine involution is dominated by the action of oxytocin alone.

22.4.3 Clinical Aspects

Premature delivery is the leading cause of perinatal morbidity and mortality and an important factor in increased medical costs and long term chronic disease. A recent study employed factor analysis to group the various demographic, clinical, histopathologic, and microbiological risk factors associated with delivery before 28 weeks' gestation [110]. Two major groups emerged, the first related to infection and inflammatory mediators and the second related to placental underperfusion and mediators of oxidative stress. While additional placental disease processes occasionally occur, other studies also support this dichotomous classification scheme [111, 112]. In the context of the discussion above, inflammatory premature labor may be understood in terms of cytokine upregulation of CRH, cortisol, and prostaglandin production while the effects of underperfusion and oxidative stress may be primarily mediated by premature maturation of the fetal hypothalamic adenal axis. Other more direct effects of these processes include chorioamnionitis that can reduce PGDH by causing necrosis of chorion laevae trophoblast and placental abruption occurring secondary to defective vascular remodeling that physically separates the placenta from the uterus and generates high levels of uterotonic thrombin [113]. Since escape from the uterus in the face of infection and severe hypoxia is often the optimal adaptive response, efforts to decrease premature delivery have been concentrated on prevention rather than treatment. However, some patients may have a genetic predisposition to exaggerated responses to uterotonic mediators in the face of treatable disease. Individualized therapy using 17-OH P4, calcium channel blockers, prostaglandin inhibitors, and possibly toll-like receptor-4 inhibitors together with more effective treatments for the underlying conditions may become increasingly utilized in the future.

22.4.4 Neoplasia

ETT is the most recently described trophoblastic malignancy [114, 115]. Like PSTT, ETT often occurs years after preceding pregnancy, but a higher percentage of ETTs (approximately two-thirds) are preceded by abortions and molar pregnancies. ETT usually presents as a well-circumscribed nodular or partially cystic lesion, often in the lower uterine segment or endocervix. Microscopically, tumor cells are arranged in cords and nests often associated with eosinophilic fibrillar hyaline material resembling keratin. Cytoplasm is clear and the cells have a higher N/C ratio than seen with PSTT (Fig. 22.2f). Serum hCG titers are intermediate

between PSTT and ChorioCA. The primary differential diagnostic considerations include squamous carcinoma, epithelioid leiomyosarcoma, and PSTT. Expression of cytokeratin 18, p63, Inhibin A and lack of smooth muscle markers and HPL are helpful ancillary techniques (Table 22.2). PSNs have a similar staining profile and may represent the precursor lesion for ETT, but are easily distinguished in most cases by their atrophic features. A proliferation index greater than 10% in an excessively cellular PSN should prompt consideration of ETT. Like PSTT, ETTs are chemoresistant, so local control is imperative. Metastases occur in 25% of cases and the mortality rate is approximately 10%.

22.5 Conclusion

Endocrine mediators play a critical role in placental development, placental function, and parturition. Future progress in uncovering new mediators and further defining the anatomic localization and regulatory networks of existing factors in the context of pregnancy-related diseases will play an important role in furthering our understanding of underlying mechanisms of perinatal biology.

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Chapter 23
Fine Needle Aspiration Cytology of Endocrine Glands

Ema A. Dragoescu and Celeste N. Powers

23.1 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 tumor 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 practised 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]. Thyroid FNAB has a low false-negative rate and a high true-positive rate providing appropriate triage of patients for surgery [13]. Parathyroid lesions, if palpable, are also amenable to FNAB. Nonpalpable lesions of both thyroid and parathyroid can be aspirated with ultrasound-guidance. Lesions of the adrenal glands are accessible to FNAB utilizing interventional radiology, without the morbidity associated with open, or even laparoscopic, biopsy techniques.

The ability to provide high-quality interpretations on FNAB material is dependent upon obtaining high-quality samples. The ability to provide a high-quality sample is influenced by many factors, such as: (1) technical skill of the operator to obtain and prepare the aspirated material; (2) assurance that the sample is representative of the lesion; and (3) the sample is sufficient in quantity and quality [13–16].

A palpable FNAB is performed using 23–27 gauge needles, with or without an attached 10 or 20 cc syringe and aspiration device (syringe holder). Using a syringe holder allows the operator to direct the needle into the lesion and apply negative pressure with one hand, and at the same time, to isolate and steady the target lesion with the nondominant hand. The needles used for FNAB should have a long bevel. The trailing edge of the needle is very effective for cutting into the tissue [13]. The needle is passed back and forth within the lesion several times to dislodge tumor cells and to collect them within the barrel of the needle. The surface tension-induced capillary action, which is high in the smaller diameter needles, together with the forward motion of the needle maintains the cellular material within the needle core [13]. The process continues just until the cellular material becomes visible in the hub of the needle. Suction is released prior to withdrawing the needle and afterwards gentle manual pressure is applied at the biopsy site. The basic principles are the same for imaging-guided FNAB. If ultrasound guidance is used, the needle must never be passed through a layer of ultrasound gel on the skin, as this gel when smeared on the slides produces an obscuring precipitation on the Romanowsky (Diff-Quik®) stain [13].

The cellular material is expelled onto a glass slide and 1–2 smear slides per pass are obtained. There are several easy-to-learn conventional smearing techniques that produce a thin layer of cells while preserving cellular morphology. The prepared slides are either fixed in ethanol (for Papanicolaou staining) or air dried (for Diff-Quik® staining). These two stains complement each other, as the Papanicolaou stain highlights best the nuclear details (nuclear contour, chromatin quality, presence of nucleoli),

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and the Diff-Quik® stain brings to attention the presence of background extracellular material, cytoplasmic details and is best suited for lymphoid lesions. The cytologic diagnostic criteria currently used have been developed using these conventional smears. Significant flaws in the smearing technique can limit the microscopic evaluation, irrespective of how much material was aspirated [13]. For this reason, some laboratories use nonsmear techniques (cell blocks and liquid-based preparations).

FNAB of a superficial, palpable lesion is a rapid and well-tolerated procedure and local anesthesia is *not* necessary. Three main reasons have been stated for not using local anesthesia in a superficial FNAB: (1) injection of local anesthetic can cause more pain than the FNAB itself; (2) the infusion of the local anesthetic can obscure anatomic details and make the target lesion more difficult to palpate; and (3) local anesthetic may cause cellular degeneration and loss of morphology [13]. For deep, nonpalpable lesions aspirated with image-guidance, local anesthesia is recommended as in these situations more time is required to reach the target. If possible, it is best to perform multiple aspirates of the same nodule to thoroughly sample the lesion and to ensure collection of a sufficient amount of material for any necessary ancillary studies. Rapid staining of selected slides using Diff-Quik® can be used to assess specimen adequacy and to allow the triage of any additional material obtained.

23.2 Thyroid

The primary indication for fine needle aspiration biopsy of the thyroid gland is the presence of a 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. A nonpalpable thyroid nodule incidentally discovered by an imaging study, such as carotid Doppler scan, computed tomography (CT), or magnetic resonance imaging (MRI), should undergo a dedicated thyroid sonographic evaluation [17]. Nodules with suspicious sonographic features or nodules greater than 1.0–1.5 cm should undergo fine needle aspiration. Suspicious sonographic features include: microcalcifications, hypoechoic solid nodules, irregular/lobulated margins, increased vascularity, nodal metastases, or signs of extracapsular spread [17, 18]. All focal thyroid nodules detected by ^{18}F-fluorodeoxyglucose positron emission tomography scan (^{18}FDG-PET) and all focal thyroid nodules hot on sestamibi scans should undergo fine needle aspiration as they have a higher risk of cancer (14–50% for FDG-PET-avid nodules and 22–60% for hot nodules on sestamibi scans) [17].

Contraindications include an uncooperative patient, severe bleeding diathesis due to the risk of hematoma formation or intrathyroidal hemorrhage with acute upper airway obstruction [17], and a suspicion of hyperthyroidism or thyrotoxicosis as it is possible to induce thyroid storm with the FNAB procedure in this setting. A recent history of anticoagulant therapy is a relative contraindication. It is usually possible to perform the procedure in this setting, especially if the smallest possible needle is used and the number of passes is limited [17]. A family history of thyroid malignancy, clinical history of head and neck or total body irradiation, the presence of cervical lymphadenopathy or vocal cord paralysis, should increase the aspirator's suspicion of malignancy [17]. The sensitivity and specificity of FNAB of the thyroid gland are both >90% [6, 15, 19].

One of the topics evaluated by the National Cancer Institute (NCI)-sponsored thyroid FNA State of the Science Conference held on October 22–23, 2007 in Bethesda, MD (http://thyroidfna.cancer.gov) was the diagnostic terminology and various classification schemes proposed for reporting thyroid FNAB results, as there is no standard reporting scheme at present time [20]. The general consensus is that a tiered classification system should be used (Table 23.1). Thyroid FNAB is a cost-effective method in the evaluation of thyroid nodules [20, 21] providing a rational approach for management and identification of patients who require surgery. Aspiration biopsy can be used to triage follicular lesions, which have a spectrum ranging from low cellularity colloid nodules to cellular follicular lesions and follicular neoplasms. It can also be used to assess diffuse inflammatory processes (i.e. thyroiditis) and to diagnose malignancies including papillary, medullary, and anaplastic carcinomas.

A thyroid FNAB sample must be adequate for evaluation, both qualitatively (technically well-preserved and well-prepared slides) and quantitatively [13]. Several authors have suggested

Table 23.1 Thyroid FNAB classification scheme (adapted from NCI thyroid FNA state of the science conference)

Diagnostic categories
Nondiagnostic (or unsatisfactory)
Cyst fluid only
Benign (or nonneoplastic thyroid)
• Colloid nodule
• Adenomatous/hyperplastic nodule
• Diffuse toxic goiter (Grave's disease)
• Thyroiditis
– Acute
– Subacute (DeQuervain's or granulomatous)
– Chronic lymphocytic (Hashimoto's)
Follicular lesion of undetermined significance (Cellular follicular lesion)
Suspicious for follicular neoplasm
Malignant
• Papillary thyroid carcinoma
• Medullary thyroid carcinoma
• Anaplastic carcinoma
• Malignant lymphoma

objective adequacy criteria for the evaluation of thyroid aspirates [22–26], typically involving counting follicular cells and cellular groups. The cellularity of a specimen is influenced by the intrinsic nature of the lesion (solid nodule or cyst) [13]. In aspirated solid nodules that have a follicular cell population with less than abundant colloid, a minimum of 5–6 groups with at least 10 follicular cells is recommended [13]. On the other hand, the diagnoses of thyroiditis and colloid nodules do not require a minimum number of follicular cells. The aspirate of a thyroid cyst will frequently yield few to no follicular cells and it should be diagnosed as "cyst fluid only" rather than unsatisfactory.

23.2.1 Colloid Nodules and Adenomatous/Hyperplastic Nodules

A dominant nodule within a multinodular thyroid is one of the most common presentations for FNAB. The majority of these nodules represent non-neoplastic lesions that yield variable amounts of colloid and follicular cells upon aspiration. The ratio of follicular cells to colloid is key to distinguishing non-neoplastic nodules (clinically, "goiters") from follicular neoplasms (Table 23.2). The greater the amount of colloid in relation to follicular cells the less likely the nodule is neoplastic [9, 10] (Fig. 23.1). The watery colloid ranges in color from pale blue to dark purple in Diff-Quik® stained smears and due to air-drying artifact cracks giving a "chicken wire" or "stained glass window" appearance. Watery colloid is much harder to recognize in alcohol-fixed preparations. It appears as a pale green film that folds on itself or can get partially detached from the slide. Thyroid follicular cells in non-neoplastic lesions are often arranged in flat, evenly spaced "honeycomb" sheets or as three-dimensional spherules with depth of focus (Figs. 23.2a and b). They are generally small, round-oval-shaped with dark, round nuclei and scant cytoplasm. Occasional nuclear size may vary from cell to cell (benign endocrine atypia). In cases with cystic degeneration, follicular cells may assume an elongated or spindle shape appearing as uniformly spaced, polarized sheets reminiscent of "tissue culture" (regressive changes) (Fig. 23.3). Another feature that helps distinguishing non-neoplastic lesions from follicular neoplasms is the presence of multiple cell types, e.g. follicular cells, some with regressive changes, Hürthle cells, lymphocytes, and macrophages. Hyperplasia, both diffuse (Graves' disease) and solitary (toxic nodule), is difficult to distinguish from a follicular neoplasm using the Papanicolaou stain alone. The Diff-Quik® stain accentuates cytoplasmic changes that have been variously named: flame cells, fire flares, and colloid suds [9, 10]. Although not pathognomonic, when prominent, these features are usually indicative of hyperplastic change and clinical correlation with radiologic study is warranted.

Cystic lesions can be particularly difficult to interpret, primarily due to the low numbers of follicular cells. The risk of malignancy in a simple, non-complex cyst is low (1–4%); however, the risk of malignancy increases to 14% in mixed solid and cystic nodules, cysts larger than 3.0 cm, and

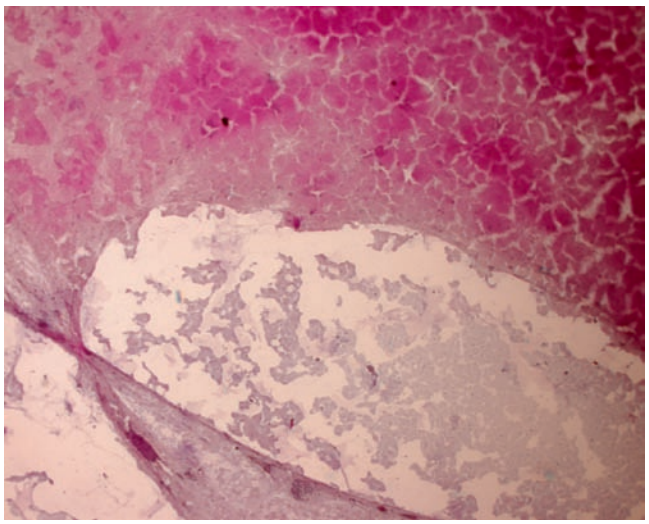

Fig. 23.1 Colloid nodule. Colloid is abundant in this aspirate. Colloid often appears as a wavy sheet that can fracture, fold on itself, or detach from the slide surface. Papanicolaou stain, ×40

Table 23.2 The spectrum of follicular lesions

Cytologic criteria	Colloid nodule	Adenomatous/hyperplastic nodule	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	Indistinct	Indistinct	Distinct
Other cell types	Occ. Histiocyte/macrophage	Occ. Lymphocytes Hemosiderin – laden macrophages	None

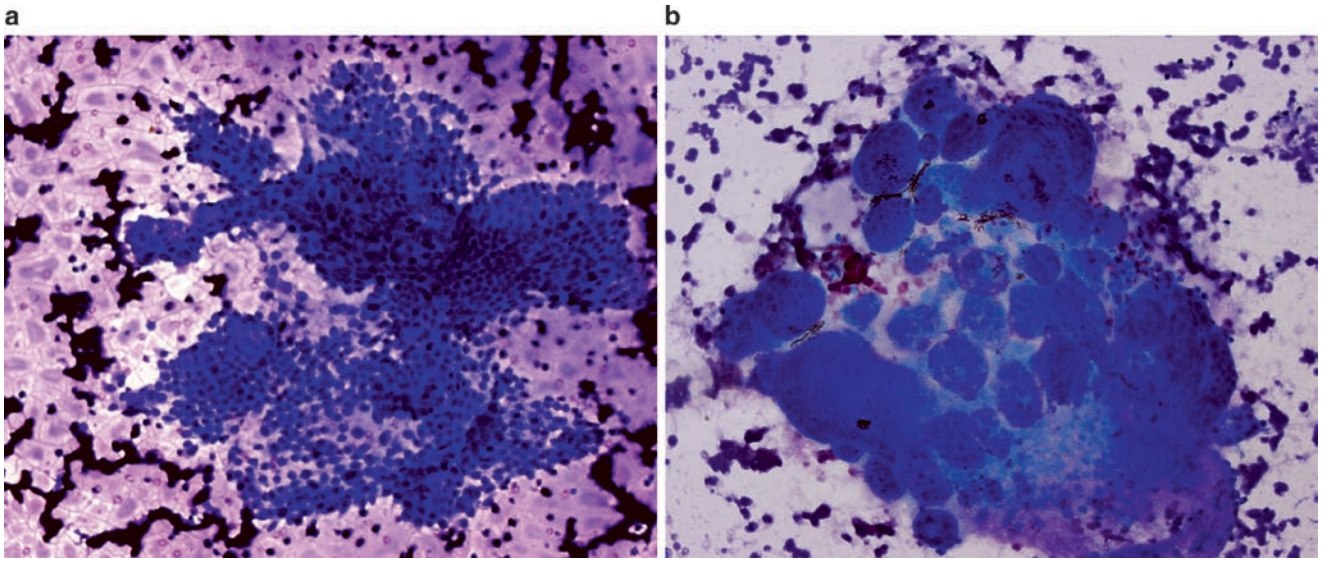

Fig. 23.2 (**a**) Non-neoplastic thyroid. Fragment of bland, evenly spaced follicular cells arranged as a flat honeycomb sheet. Note the dark purple colloid in the background with a "shattered glass" appearance. Diff-Quik® stain, ×200. (**b**) Non-neoplastic thyroid. Thyroid follicles are often aspirated as intact variably sized three-dimensional spherules. Diff-Quik® stain, ×200

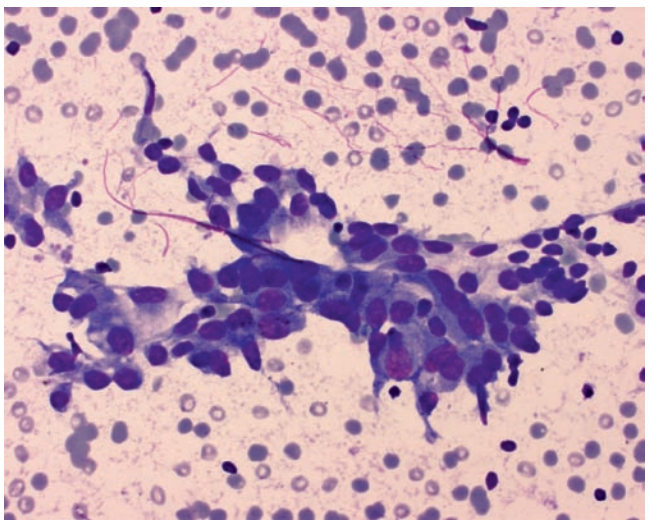

Fig. 23.3 Regressive changes. If cystic degeneration occurs in a non-neoplastic thyroid, follicular cells can become spindled, with a "tissue culture" appearance. Diff-Quik® stain, ×400

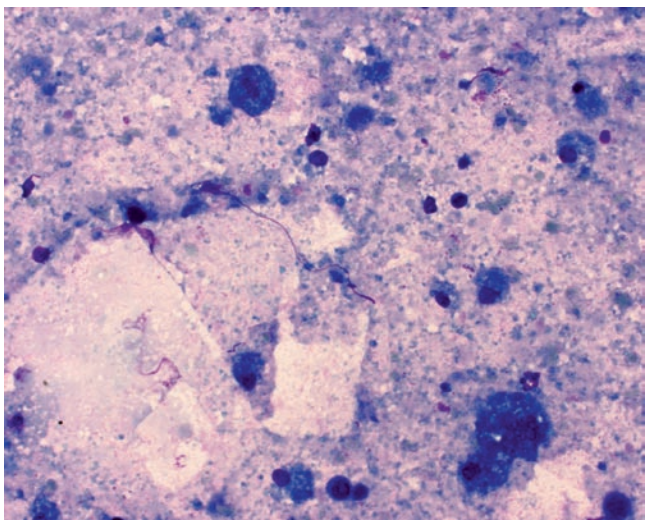

Fig. 23.4 Cystic degeneration in non-neoplastic thyroid. Histiocytes, hemosiderin-laden macrophages, and cholesterol crystals are features that indicate the presence of cystic changes. Diff-Quik® stain, ×400

recurring cysts [13]. Most of these lesions are not true cysts, but rather areas of cystic degeneration in a background of nodular goiter due to prior hemorrhage. It is recommended to drain the cyst and aspirate any residual palpable mass or solid component visible on ultrasound [13]. The cyst fluid can be processed fresh into cytospin slides or collected in vials for liquid-based preparations. The features that indicate cystic change include abundant hemosiderin-laden macrophages, degenerative/regressive changes of follicular cells, and in long-standing cases dystrophic calcification and cholesterol crystals (Fig. 23.4). Cystic degeneration can also occur in thyroid malignancies, particularly in 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].

23.2.2 Thyroiditis

Inflammatory diseases usually result in diffuse involvement of the thyroid gland, rather than a distinctive nodule (Table 23.3). Acute thyroiditis is rarely encountered on aspiration since 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, 27, 28] (Fig. 23.5). Subacute or granulomatous (DeQuervain's) thyroiditis is usually self-limited, resolving over several weeks and therefore, is not commonly aspirated. FNAB of this condition can be painful [13]. As its very name implies, the consistent finding is one of degenerated follicular cells with an inflammatory component of histiocytes, multinucleated giant cells and granulomas [9, 10, 28, 29, 30, 31] (Fig. 23.6). Colloid is usually scant, while fibrosis and cellular debris may predominate. The inflammatory condition most often aspirated is chronic lymphocytic (Hashimoto's) thyroiditis, which can present as a distinct nodule in a diffusely enlarged thyroid gland. Histologically, it is characterized by a diffuse lymphocytic infiltrate of the thyroid gland with atrophy of the thyroid follicles and Hürthle cell metaplasia. Similar constellation of findings is present on FNAB material. The aspiration smears are moderately cellular with groups of follicular cells and scant to absent colloid. A heterogeneous population of lymphocytes mixed with plasma cells is the predominant inflammatory component in the background or interspersed between the groups of follicular cells. The lymphoid population consists of a mixture of

Table 23.3 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)	Multinucleated giant cells	Hürthle cells
	Histocytes, fibroblasts (late stage)	Epithelioid histocytes	Polymorphous population of lymphocytes, germinal centers, plasma cells
		Granulomas	
		Mixed inflammatory cells	
Background elements	Bacteria, fibrin	Debris	Lymphoid tangles

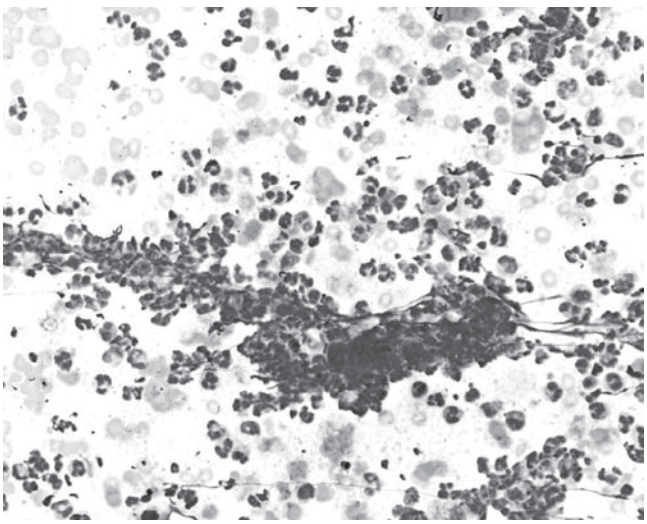

Fig. 23.5 Acute thyroiditis. Numerous neutrophils, fibrin, and cell debris are the predominant features in this diffuse inflammatory process. Papanicolaou stain, ×200

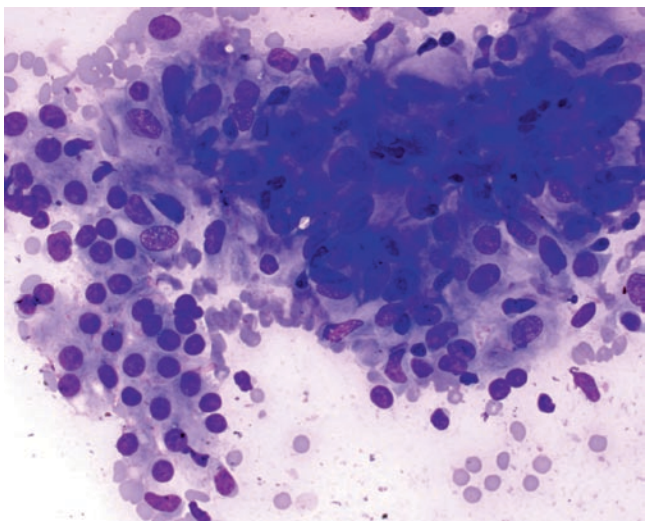

Fig. 23.6 Subacute thyroiditis. Aspirate smears show a cluster of follicular cells adjacent to a group of epithelioid histiocytes. Chronic inflammatory cells may be seen in the bloody background devoid of colloid. Diff-Quik® stain, ×400

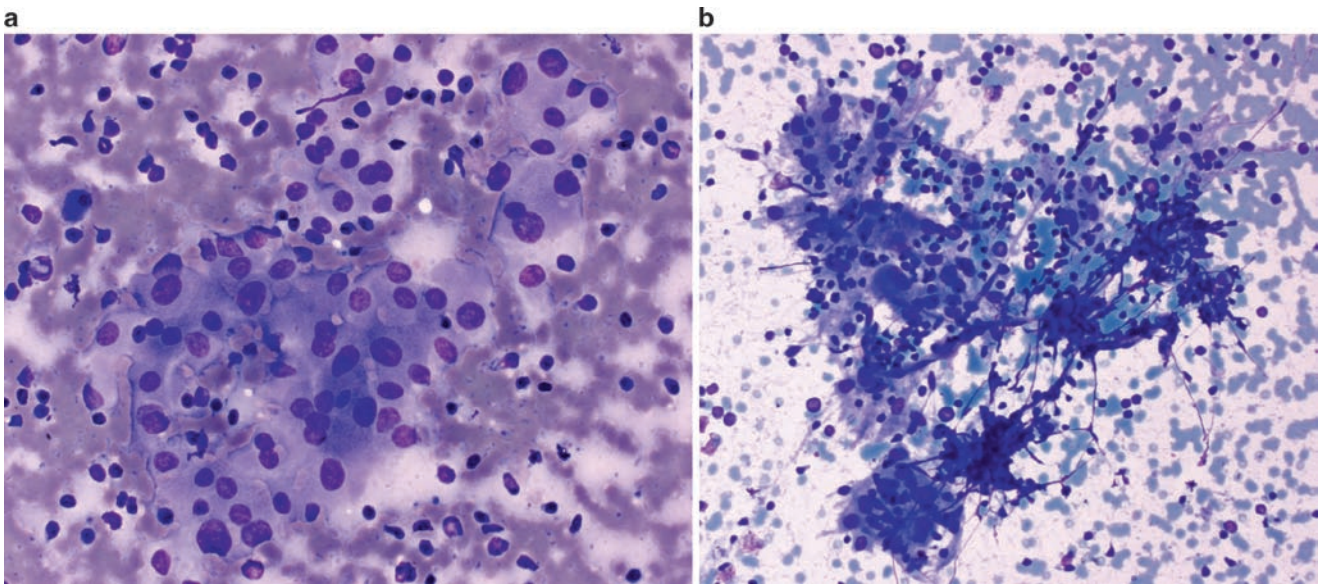

Fig. 23.7 (**a**) Hashimoto's thyroiditis. Moderate to high cellularity smear consisting of a polymorphous population of lymphocytes and a group of follicular cells with Hürthle cell changes. Lymphocytes are present in the background and also mixed within the group of follicular cells. Diff-Quik® stain, ×400. (**b**) Hashimoto's thyroiditis. Lymphohistiocytic aggregates and lymphoid tangles are often seen in chronic lymphocytic thyroiditis. Diff-Quik® stain, ×200

small, mature lymphocytes, intermediate-sized centrocytes, and centroblasts, large immunoblasts, as well as tingible body macrophages and lymphohistiocytic aggregates (Fig. 23.7a). Lymphoid cells are fragile and during the smear preparation can get distorted appearing as lymphoid tangles and disrupted lymphocyte DNA (Fig. 23.7b). Hürthle cells are often present as cells scattered singly or in small groups. A diagnostic problem may occur if a palpable Hürthle cell nodule develops, since selective aspiration of this non-neoplastic proliferation may result in a diagnosis of Hürthle cell neoplasm. 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, i.e. serum autoantibodies, can help confirm the clinical and cytologic impression.

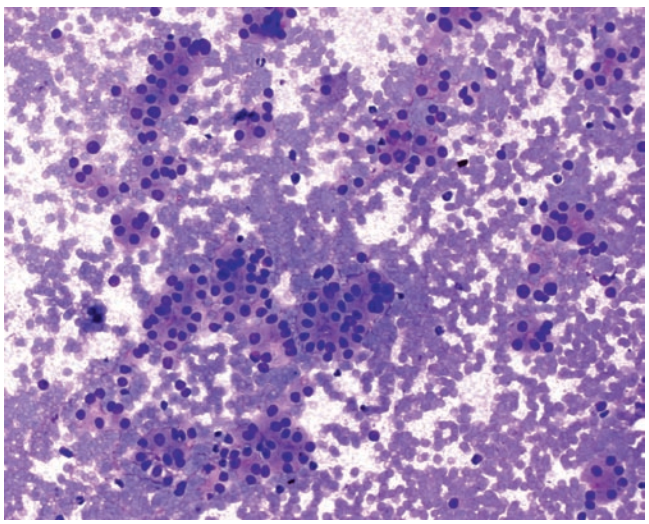

Fig. 23.8 Follicular neoplasm. Highly cellular aspirate composed of a uniform population of follicular cells arranged in microfollicles. Colloid is conspicuously absent. This case requires surgical excision, as the distinction between a follicular adenoma and follicular carcinoma cannot be made on cytology. Diff-Quik® stain, ×200

23.2.3 Cellular Follicular Lesions

Thyroid nodules that yield hypercellular aspirates with high follicular cells to colloid ratio are best classified as cellular follicular lesions. This is a generic category that encompasses non-neoplastic lesions (cellular adenomatous nodule in a multinodular goiter, or nodular hyperplasia) and follicular neoplasms (follicular adenoma, follicular carcinoma, or follicular variant of papillary carcinoma) [9, 10]. This is the most challenging area of thyroid FNAB. Clinicians should correlate cytology findings with their clinical impression and ultrasound findings in order to determine whether close clinical follow-up, repeat FNAB, or surgical excision is indicated.

High cellularity aspirates consisting of a monotonous population of follicular cells with minimal or absent colloid are very worrisome for follicular neoplasms. The follicular cells are arranged in large irregular three-dimensional groups with nuclear crowding and overlapping, trabeculae, repetitive microacini or microfollicles [20] (Fig. 23.8). A microfollicle

is defined as a group of less than 15 cells arranged in circle that is at least two-thirds complete [32]. The neoplastic cells have increased nuclear to cytoplasmic ratios and small yet distinct nucleoli [9, 10, 12, 33]. Thyroid follicular neoplasms cannot be definitively separated into follicular adenomas and follicular carcinomas using FNAB alone; this distinction requires histologic evaluation for the presence of capsular and vascular invasion. Therefore, a diagnosis of follicular neoplasm on FNAB needs to be followed by surgical excision.

The same holds true for Hürthle cell neoplasms. Hürthle cells appear in cytologic smears as large, polygonal cells with well-defined cell borders, abundant granular cytoplasm, and round nuclei with large, prominent nucleoli (Figs. 23.9a and b). They are frequently seen in many non-neoplastic thyroid lesions as isolated cells and represent localized metaplastic changes of follicular cells or un-encapsulated nodules [20]. However, when a pure population of Hürthle cells is encountered on FNAB with minimal or absent colloid in the background, a diagnosis of Hürthle cell neoplasm needs to be suspected. In this situation, the Hürthle cells can be arranged as flat sheets, three-dimensional groups, or dispersed as single cells. Similar to follicular neoplasms, Hürthle cell neoplasms cannot be further differentiated into benign or malignant on cytomorphology alone and surgical excision is indicated for a definitive diagnosis [10, 12, 34]. Hürthle cell lesions are particularly problematic for two reasons. First, they can show significant cytologic atypia (increased nuclear to cytoplasmic ratio, cellular pleomorphism, random nuclear enlargement, binucleation) yet behave as benign lesions [20]; the converse is also true, relatively bland Hürthle cell nodules may be diagnosed as carcinoma when excised. Second, non-neoplastic Hürthle cell proliferations in Hashimoto's thyroiditis may produce a discrete nodule that, when aspirated, yields a pure population of Hürthle cells that is misdiagnosed as a neoplasm.

23.2.4 Malignant Neoplasms

FNAB is the diagnostic procedure for malignant neoplasms of the thyroid with well-known and distinctive cytologic criteria for the major malignancies (Table 23.4).

Papillary thyroid carcinoma (PTC) is the most frequent thyroid malignancy and specific cytologic criteria allow for a very accurate diagnosis of this cancer [9, 10, 35, 36]. The major diagnostic criteria are the nuclear features, best appreciated on Papanicolaou stain. Nuclei in PTC are enlarged, oval, elongated with a fine, pale, or powdery nuclear chromatin, thickened nuclear membranes, and can demonstrate nuclear grooves and intranuclear cytoplasmic inclusions (Figs. 23.10a and b). Nucleoli are visible, but small, and characteristically are eccentrically placed. Nuclear grooves can extend along the longest axis of the

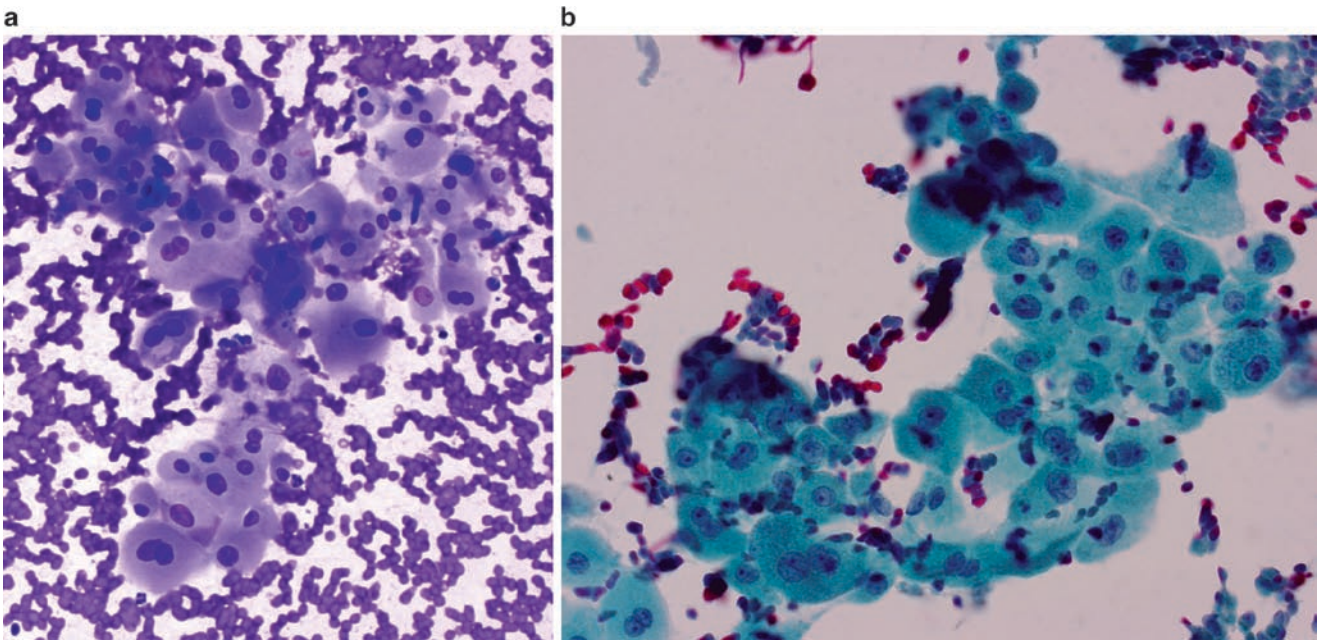

Fig. 23.9 (**a**) Hürthle cell neoplasm. Cellular aspirate with a pure population of Hürthle cells characterized by abundant, finely granular cytoplasm and round nuclei with prominent nucleoli. Binucleation is not uncommon. Colloid is sparse to absent in this lesion. Diff-Quik® stain, ×200. (**b**) Hürthle cell neoplasm. Hürthle cell neoplasms often show variable cell size and nuclear atypia; however, the biologic behavior of these lesions cannot be predicted by cytologic atypia. Note the abundant granular cytoplasm characteristic of these cells, due to presence of increased number of mitochondria. Papanicolaou stain, ×400

Table 23.4 Cytologic criteria of thyroid carcinomas

Cytologic criteria	Papillary carcinoma	Medullary carcinoma	Anaplastic carcinoma
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	Variable morphology: plasmacytoid,	Extreme cytologic atypia
	Squamoid cytoplasm	Hürthle-like, spindle-shaped carcinoid-like	High N/C ratio
	Nuclear grooves and inclusions	Neuroendocrine chromatin	Prominent nucleoli
	Powdery chromatin	Neurosecretory granules	Atypical mitoses
Background Elements	Multinucleated giant cells	Amyloid	Neutrophils
	Psammoma bodies		Debris
	Occ. cystic degeneration		Necrosis

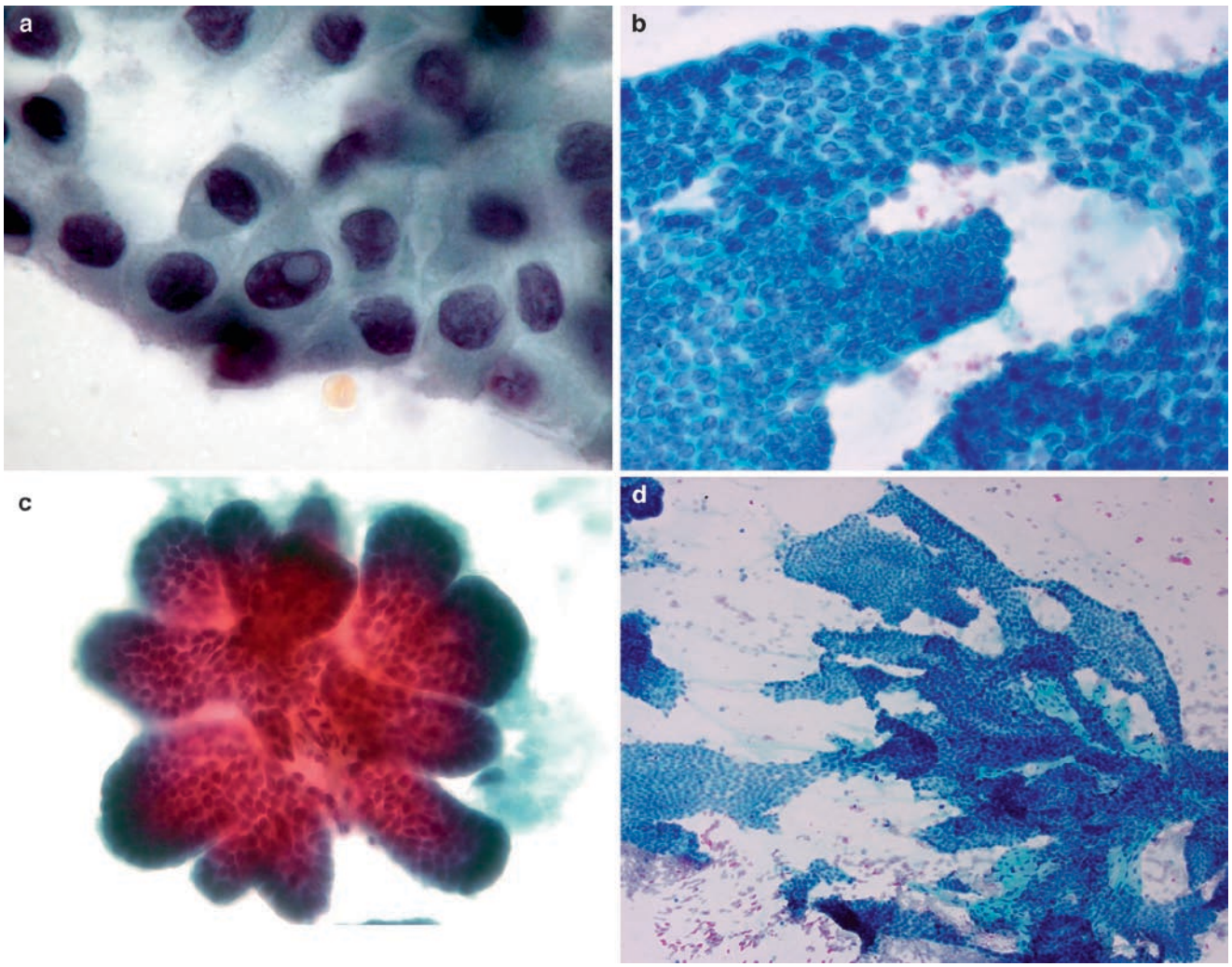

Fig. 23.10 (**a**) Papillary carcinoma. Neoplastic follicular cells with irregular nuclear membranes and squamoid cytoplasm. Note the sharply demarcated intranuclear cytoplasmic inclusion with the same color as the cell cytoplasm. Papanicolaou stain, ×400. (**b**) Papillary carcinoma. Large sheets of neoplastic follicular cells. Papanicolaou stain highlights the characteristic nuclear features of this neoplasm: elongated, overlapped nuclei with pale, powdery chromatin, visible nuclear grooves, and small, eccentrically placed nucleoli. Papanicolaou stain, ×400. (**c**) Papillary carcinoma. Papillary groups with fibrovascular cores covered by neoplastic follicular cells may be aspirated intact and appear as three-dimensional structures. Papanicolaou stain, ×200. (**d**) Papillary carcinoma. Highly cellular aspirate with detached large folded sheets of follicular cells and naked fibrovascular cores. Papanicolaou stain, ×100

nucleus or can manifest as short invaginations of the nuclear contours. Strict criteria need to be applied in the identification of intranuclear cytoplasmic inclusions to separate them from artifactual clearing of the nucleus. Classic intranuclear cytoplasmic inclusions have a sharp outline and the same color as the cytoplasm. The last two nuclear features, nuclear grooves and intranuclear cytoplasmic inclusions, can be seen in many other thyroid conditions (multinodular goiter, Hashimoto's thyroiditis, Hürthle cell neoplasms, medullary thyroid carcinoma), therefore the diagnosis of PTC should not rest solely on these two features, but should be supported rather by several criteria in one given case [20]. Minor criteria useful in supporting the diagnosis of PTC pertain to low-power architecture of the aspirate smears, cytoplasmic qualities, and additional background elements. FNAB of a PTC yields in general cellular aspirates, with tumor cells arranged in obvious papillary groups or in syncytial monolayers (Figs. 23.10c and d). The papillary groups appear as elongated, three-dimensional clusters with visible fibrovascular cores. The cells lining the fibrovascular core will be sheared from this core during slide preparation (degloving) resulting in large, folded and overlapped sheets of cells and naked fibrovascular cores. The cytoplasm of follicular cells in PTC is described occasionally as being dense, squamoid. Background elements suggestive of PTC include dense ropy or "bubble gum" colloid, multinucleated giant cells, and psammoma bodies. The ropy colloid is best appreciated on Diff-Quik® stain as dense, vivid metachromatic, thick, acellular material.

Medullary carcinoma of the thyroid (MCT) is a malignant neoplasm of parafollicular C-cells and occurs sporadically or as part of a familial syndrome. An accurate preoperative diagnosis of MCT by FNAB allows for further investigations to exclude a familial syndrome, planning for total thyroidectomy with central lymph nodes dissection, and measurement of baseline serum calcitonin levels for postoperative follow-up [37]. Aspirated material is cellular, consisting predominantly of dispersed single cells with occasional loosely cohesive aggregates noted. Tumor cells have variable morphology from case to case and even within the same aspirate: epithelioid cells with eccentric nuclei (plasmacytoid appearance), small round-oval cells, spindle-shaped cells, or Hürthle cell-like cells [9, 10, 38–41] (Figs. 23.11a and b). Tumor cells have abundant eosinophilic granular cytoplasm due to red perinuclear granules best appreciated on the Diff-Quik® stain and corresponding to the neurosecretory granules identified by electron microscopy. Characteristically, nuclei have a finely granular chromatin with inconspicuous nucleoli, imparting a neuroendocrine appearance. Moderate nuclear pleomorphism is common in this tumor and bi- and multinucleated cells can be seen. Intranuclear cytoplasmic inclusions can be seen in MCT. Amyloid, when present, appears as an amorphous pink or magenta extracellular material resembling the dense, ropy colloid characteristic of PTC. The absence of amyloid in FNAB material does not exclude the diagnosis of MCT. The presence of amyloid should be confirmed with a Congo red stain. Confirmatory immunohistochemical stains on cell block (positive for calcitonin, calcitonin gene-related peptide,

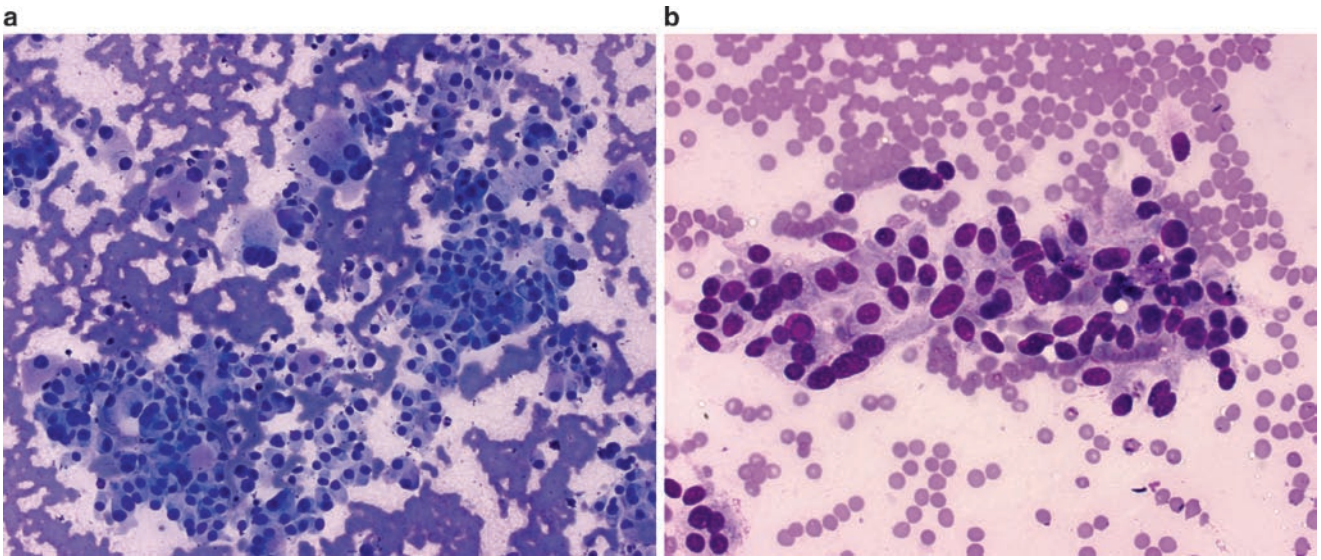

Fig. 23.11 (**a**) Medullary carcinoma. Aspirates of this malignancy are highly cellular with a discohesive population of cells with variable pleomorphism. Plasmacytoid cells with eccentrically placed nuclei mixed with bi- and multinucleated cells are seen. Some of the cells have abundant eosinophilic granular cytoplasm with distinct red (on Diff-Quik®) perinuclear granules corresponding to neurosecretory granules. Amyloid, visible in the lower left hand corner, appears as an amorphous pink material. Diff-Quik® stain, ×200. (**b**) Medullary carcinoma. The spindle cell variant of medullary carcinoma consists of loosely cohesive groups of cells with scant to moderate cytoplasm and elongated oval nuclei. Note the presence of an intranuclear cytoplasmic inclusion, which may be seen in medullary carcinoma. Diff-Quik® stain, ×400

CEA, and chromogranin, and negative for thyroglobulin) are much more helpful in confirming the diagnosis. The predominant dispersed pattern with variable cell morphology, neuroendocrine-type nuclear chromatin pattern, red intracytoplasmic granules are distinctive cytologic features characteristic of MCT. Because of its highly variable morphology, differential diagnosis is broad and includes other thyroid neoplasms (such as Hürthle cell neoplasm, PTC, or even anaplastic carcinoma), plasmacytoma, paraganglioma, metastatic tumors, as well as neural lesions, desmoplastic melanoma, and sarcomas for spindle cell variant of MCT [20, 37, 42].

The diagnosis of anaplastic thyroid carcinoma by FNAB is usually not difficult, as the aspirated material is highly cellular with extremely pleomorphic epithelioid, spindle-shaped, and multinucleated giant malignant cells usually accompanied by a neutrophilic infiltrate, atypical mitoses, and necrosis in the background [9, 10, 20, 43–45] (Fig. 23.12). The nuclei have irregularly distributed, coarsely granular chromatin, irregular nuclear contours, and multiple nucleoli. Anaplastic, giant tumor cells engulfing neutrophils can be seen (leukophagocytosis). The diagnosis is usually suspected clinically since these carcinomas present emergently in elderly patients as rapidly enlarging masses that often compromise breathing. This presentation is also typical of high-grade malignant lymphomas of the thyroid (Fig. 23.13); however, 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 extension of tumors from nearby structures, particularly squamous cell carcinoma, and less commonly, adenoid cystic carcinoma of the trachea [46]. Distant metastases to the thyroid gland are rare. The most common primary sites that have been reported to metastasize to the thyroid gland are: kidneys, lungs, breasts, colon, and malignant melanoma [47]. Poorly differentiated metastases may be challenging to distinguish from anaplastic carcinoma. Renal cell carcinoma may mimic thyroid neoplasms, when present in papillary configuration and individual cells may be particularly difficult to distinguish from histiocytes commonly encountered in thyroid cystic lesions. Cytologic clues include the delicate, often frayed cytoplasm and microvesicular smear background of renal cell carcinomas. 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.

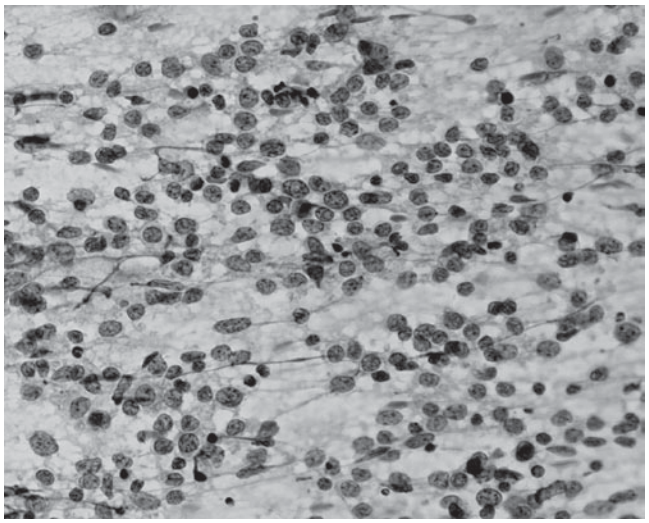

Fig. 23.13 Non-Hodgkin lymphoma. Highly cellular aspirate with monomorphic population of neoplastic lymphocytes. DNA artifact manifested as strings of nuclear material flowing in the direction of the smear is common due to fragility of the tumor cells. Papanicolaou stain, ×200

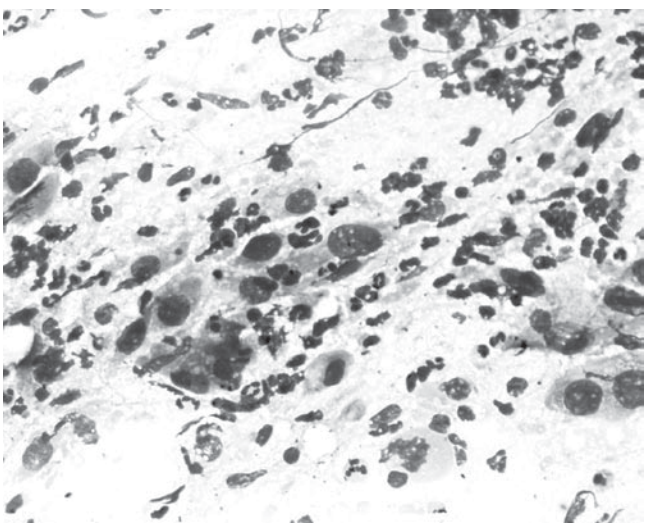

Fig. 23.12 Anaplastic carcinoma. Cellular aspirate with numerous pleomorphic malignant cells with high nuclear to cytoplasmic ratios. Necrosis and neutrophils are frequent background elements. Diff-Quik® stain, ×200

The pathologist needs to be aware that FNAB can create changes in the thyroid that can complicate histologic interpretation on the excision specimen [48–53]. These changes are most frequently known as "worrisome histologic alterations following fine-needle aspiration of the thyroid" or WHAFFT. The incidence of these changes varies, but is usually reported to be less than 40%. Typically, this occurs when larger diameter needles are used and/or multiple, separate passes are performed. Histologic 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. These changes are, in general, confined to the needle tract area. Of all these alterations, the most significant are infarction and capsular distortion, as

this can make the histologic diagnosis of malignancy and histologic evaluation for true capsular invasion more challenging. Awareness of previous FNAB results is an essential component of the histologic diagnosis of a lesion with extensive post-FNAB infarction. Of all thyroid lesions, Hürthle cell neoplasms are most likely to become infarcted, either spontaneously or as the result of FNAB.

23.2.5 Ancillary Studies

Ancillary studies are necessary in the following situations: (1) to characterize suspected thyroid malignancies, in particularly medullary thyroid carcinoma, anaplastic carcinoma, and malignant lymphoma; (2) when a parathyroid lesion is suspected; and (3) in suspected cases of metastatic thyroid carcinoma to the lymph nodes [47]. The most commonly used and widely available test is immunohistochemistry (IHC) on cell block material, preferably with one pass dedicated entirely for the cell block. IHC can be performed on other type of preparations (such as cytospin slides, direct smears, prefixed monolayer preparations), but each laboratory should carefully validate the protocol, reagents, and controls for these types of specimens as the reactivity may differ from formalin-fixed paraffin embedded histologic sections obtained from a cell block [47]. The panel of antibodies to use depends on the cytologic features and suspected diagnosis. When medullary thyroid carcinoma is suspected, a serum calcitonin level can also be measured. It is important to keep in mind that anaplastic thyroid carcinoma loses expression of thyroglobulin and TTF-1, while pan-cytokeratin is only focally expressed. When a metastasis to the thyroid gland is suspected (and confirmed with negative thyroglobulin and TTF-1 staining) an extended IHC panel needs to be used, depending on the prior history of malignancy, cytomorphologic features of the current thyroid FNAB sample, and radiologic imaging findings.

Another ancillary study that is widely used and available is flow cytometric immunophenotyping in suspected cases of malignant lymphoma [47]. The fresh material needs to be collected in supportive media (such as RPMI). Preferably, one dedicated pass should be included, to obtain an adequately cellular sample for this study. The results of flow cytometric analysis should be interpreted with caution in the case of Hashimoto's thyroiditis [54, 55].

At this time, the use of molecular studies on thyroid FNAB samples in clinical practice is limited, however, it is expected that their utilization in the future will increase, as standardized protocols with clinical validation are developed [47]. These studies can be very useful in cases diagnosed as indeterminate/suspicious on cytologic grounds alone. This is the most problematic group to manage clinically, and the availability of ancillary studies that can reclassify these cases into benign or malignant is needed [47]. From all the molecular markers reported to be associated with thyroid carcinomas, the most promising results are in detection of *BRAF* mutation and *RET/PTC* chromosomal rearrangements on FNAB material [56–58].

23.3 Parathyroid

The primary indication for FNAB of the parathyroid gland is the presence of a palpable neck mass [10, 59]. Usually, a parathyroid lesion is but one possible diagnosis in a clinical differential that includes thyroid and possibly lymph node lesions. Ultrasound-guided FNAB of suspected parathyroid tissue in patients with known primary hyperparathyroidism is increasingly used as a safe and effective preoperative localization technique for patients that are candidates for minimally invasive parathyroidectomy [60–64]. This technique is particularly useful in patients with prior failed surgery, where full neck exploration cannot be performed to differentiate parathyroid adenomas from posterior thyroid nodules, or in the case of parathyroid adenomas with atypical location (i.e. intrathyroidal, submandibular) [64–66]. The ultrasound-guided FNAB can be combined with on-site rapid parathyroid hormone (PTH) assay to confirm localization of the parathyroidal tissue [65]. When used preoperatively to localize the parathyroid tissue, in conjunction with measurement of PTH in the aspirate, ultrasound-guided FNAB is highly sensitive (91%) and specific (95%) [63].

It is very important to be able to recognize the normal cytomorphology of the parathyroid cells, as there is considerable overlap with thyroid follicular cells and in some cases even with C cells. This is the first, and frequently the critical step, in narrowing the differential diagnosis, especially in cases in which parathyroid tissue is not suspected clinically and the specimen is submitted as thyroid nodule. The aspirated material from a parathyroid gland yields moderately to highly cellular smears with no colloid in the background [67]. The cells are arranged in cohesive clusters or dispersed singly. The cohesive clusters show nuclear crowding and overlap; frequently, the cells are arranged in microfollicles or papillary groups with prominent fibrovascular cores, thus mimicking a thyroid cellular follicular lesion or papillary thyroid carcinoma (Figs. 23.14a and b). The parathyroid cells are smaller than the thyroid cells; however, in a given specimen this size comparison is not always available. The nuclei are round, hyperchromatic, with stippled chromatin characteristic of neuroendocrine cells. Parathyroid cells have a granular cytoplasm with fine cytoplasmic vacuolization, a very useful clue in distinguishing them from thyroid follicular cells. The cytoplasm of parathyroid chief cells is fragile

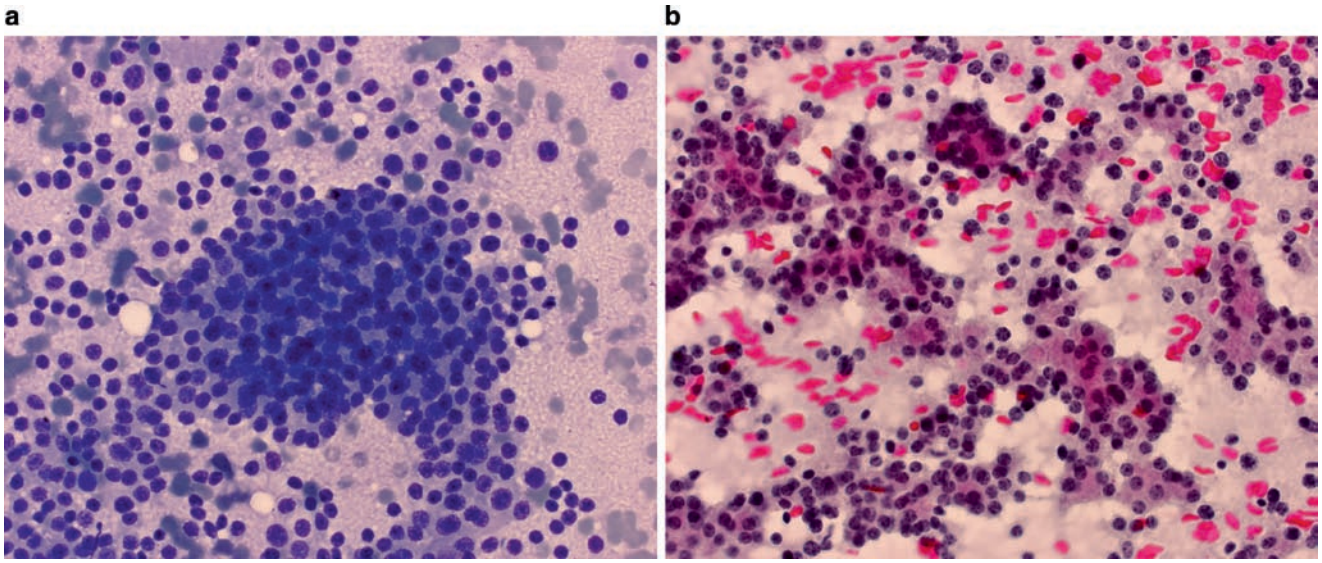

Fig. 23.14 (**a**). Parathyroid gland. Aspirates from parathyroid lesions are highly cellular with a dispersed population of small delicate cells with round nuclei and indistinct nucleoli. The fragile cytoplasm is easily disrupted during smear preparation creating a frothy, finely vacuolated background with numerous stripped nuclei. Diff-Quik® stain, ×400. (**b**) Parathyroid gland. In aspiration smears parathyroid chief cells appear dispersed as single cells or in crowded groups; occasionally they can form microfollicles. This pattern can be confused with a thyroid follicular neoplasm. Papanicolaou stain, ×400

and easily disrupted during smear preparation; as a result, numerous stripped, naked nuclei may be scattered in delicate frothy background. These naked nuclei can be confused with lymphocytes, leading to an erroneous diagnosis of Hashimoto's thyroiditis [68, 69]. Oxyphilic cells can predominate in some cases and can be *confused* with thyroid Hürthle cells leading to an erroneous diagnosis of Hürthle cell neoplasm [70]. Oxyphilic parathyroid cells have small, dark nuclei different from thyroid Hürthle cells, which have large nuclei with prominent nucleoli. This feature combined with the presence of numerous naked nuclei in the background should point towards the possibility of an oncocytic parathyroid adenoma, although the distinction from a thyroid Hürthle cell neoplasm may not be that easy in any given case [70]. Intranuclear cytoplasmic inclusions have been occasionally reported in both parathyroid adenoma and carcinoma [67, 71]. Their presence in a cellular aspirate can lead to the diagnosis of papillary thyroid carcinoma or even medullary carcinoma. Some reports have indicated that parathyroid adenomas that had undergone preoperative FNAB develop a fibrotic reaction that involves the adjacent structures and makes surgical excision more difficult with risk of incomplete surgical removal and injury to the recurrent laryngeal nerve [66]. In addition, the presence of fibrous bands within the tumor can lead to confusion histologically with a parathyroid carcinoma. The likelihood of inducing fibrosis within an adenoma increases with multiple needle passes and/or usage of large diameter needles [64, 66].

As with follicular lesions of the thyroid, parathyroid hyperplasia, adenoma, and carcinoma cannot reliably be distinguished on FNAB [10, 59, 72]. Given the significant overlap in cytomorphology, immunochemistry for PTH, thyroglobulin and even calcitonin are necessary as they reliably distinguish parathyroid from thyroid follicular cells and C cells, respectively [62, 67]; parathyroid cells are also positive for chromogranin and synaptophysin. Immunohistochemical stains are more reliable on cell block material, thus needle rinses and minicores should be processed as such when possible. Alternatively, needle rinses using minimal volume to avoid dilution, can be sent for chemical analysis to measure PTH levels, preferably with a comparative serum sample [73]. Cases with scant aspiration material, conflicting morphology, and limited clinical and radiologic information should be given a more generic interpretation.

When parathyroid cysts are aspirated water-clear fluid is obtained [10, 74]. 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 PTH will confirm the diagnosis. Parathyroid carcinoma should be suspected when aspirates show pronounced cellularity, increased numbers of single cells with marked cellular anaplasia, and necrosis [75, 76]. However, the definitive diagnosis of carcinoma rests on irrefutable histologic evidence of local invasion or metastasis.

23.4 Adrenal

Image-guided FNAB of the adrenal glands is indicated in patients with known history of malignancy that are suspected to have an adrenal metastasis [77]. The sensitivity and specificity of adrenal gland FNAB for the diagnosis of metastatic

malignancy are both >90% [78, 79]. However, for patients with functioning, clinically apparent adrenal masses and patients with incidentally discovered adrenal masses, FNAB is not routinely performed. The number of clinically inapparent adrenal masses ("incidentalomas") detected by abdominal imagining will continue to increase as these techniques have improved resolution and are more and more frequently used. The two main questions that need to be addressed once an incidentaloma is detected are: (1) is this a functioning adrenal tumor?, and (2) is this a malignant tumor (metastasis or primary adrenal malignant tumor)? [80]. The NIH state-of-the-science consensus conference concluded that all patients with adrenal incidentalomas should undergo first laboratory analyses to assess functioning capabilities of the adrenal mass (1-mg dexamethasone-suppression test, measurement of plasma-free metanephrines, serum potassium, and plasma aldosterone concentration/plasma renin activity ratio) [81]. Unfortunately, hormonal analysis cannot distinguish between a benign or malignant primary adrenal tumor [77]. Imaging characteristics of the mass should also be incorporated in the management process (size less than 4.0 cm, homogeneous mass with low attenuation value (less than 10 Hounsfield units) on CT scan can be monitored clinically) [81]. For all functioning, clinically apparent adrenal cortical tumors, all tumors with biochemical evidence compatible with pheochromocytoma, and tumors greater than 6.0 cm surgical excision should be considered, without the use of preoperative FNAB. There is a group of patients with nonfunctioning adrenal tumors in which the surgical planning may require a preoperative FNAB (tumors between 4.0 and 6.0 cm in size, tumors that show worrisome signs on CT-scan, such as irregular enhancement with intravenous contrast, areas of hemorrhage and central necrosis, local invasion, enlarged lymph nodes) [82]. Some authors have advocated the use of image-guided FNAB in conjunction with MRI findings as the cost-effective strategy of choice in the management of nonfunctional adrenal masses greater than 2.0 cm in size [80].

Image-guided FNAB of the adrenal glands can be performed percutaneously (CT-guided or ultrasound-guided) using spinal-type 21–23 gauge needles and local anesthesia or endoscopic ultrasound (EUS)-guided, preferably with a cytopathologist on-site for rapid interpretation of the specimen. EUS-guided FNAB of the left adrenal gland has been reported to be safe and accurate when compared with the percutaneous approach, as the only organ traversed by the needle is the gastric wall [83–86]. EUS-guided FNAB of the right adrenal gland is much more challenging due to its retrocaval location. However, there are a few reported cases of successful transduodenal approach using a curvilinear endoscope with a 22-gauge needle [83, 84]. Relative contraindications due to risk of hematoma formation include a recent history of anticoagulant therapy or the presence of a bleeding diathesis. One absolute contraindication is suspicion of pheochromocytoma, due to the risk of an induced catecholamine crisis with blood pressure alterations and/or fatal hemorrhagic complications. These serious complications have been described during percutaneous cutting-needle biopsy of a pheochromocytoma [87]. Because all incidentally discovered adrenal masses undergo baseline hormonal analysis, FNAB of an unsuspected functioning pheochromocytoma is extremely rare. Still, there are unsuspected nonfunctioning pheochromocytomas that have undergone FNAB using 21–23 gauge needles without incident [77, 84, 88]. FNAB of the adrenal glands has a low rate of complications (4.3%), with rare reported cases of self-limited, asymptomatic pneumothorax or adrenal hematoma which have resolved spontaneously [77].

Like the parathyroid gland, the normal adrenal cortex, adrenal cortical hyperplasia, and cortical adenoma are, cytologically, identical in appearance and cannot be differentiated on cytologic material alone. Knowledge of the imaging findings is, therefore, critical (Table 23.5). The FNAB specimens are typically cellular with uniform adrenal cortical cells dispersed singly, in acinar groups, or small aggregates, with scattered stripped nuclei in the background. Adrenal cortical cells are polygonal, with delicate, finely vacuolated cytoplasm, poorly defined cellular borders, centrally placed round-to-oval nuclei with inconspicuous nucleoli. The cytoplasm detaches easily from the nucleus during smear preparation and due to the lipid content lends a microvesicular (or bubbly) appearance to the background (Fig. 23.15). Mitotic figures and necrotic debris are absent. Finding endocrine atypia manifested as focal anisonucleosis with occasional

Table 23.5 Cytologic criteria of selected adrenal lesions

Cytologic criteria	Cortical adenoma	Cortical adenocarcinoma	Pheochromocytoma
Cellularity	Moderate-high	Moderate-high	High
Pattern	Discohesive	Predominantly discohesive	Discohesive
	Loose groups	Loose groups	Loose groups
	Stripped nuclei	Stripped nuclei	
Tumor cells	Bland	Bland to moderate atypia	Marked polymorphism
		Mitoses	Distinct nucleoli
			Occ. mitoses
Background	Microvesicular (lipid)	Microvesicular (lipid) Occ. Necrosis	Bloody

enlarged, hyperchromatic nuclei has no significance [77, 79, 85, 86, 89, 90]. Cytologic evidence, worrisome for an adrenal cortical carcinoma, includes the presence of necrosis, diffuse cytologic atypia (nuclear pleomorphism and increased nuclear to cytoplasmic ratio, coarsely granular chromatin, multiple prominent and bizarre nucleoli), and mitotic figures, especially atypical forms [77, 86, 91]. It is not difficult to classify these cases as malignant; however, they are sometimes difficult to distinguish from other malignancies, in particular renal cell carcinoma or hepatocellular carcinoma. Collecting a cell block for ancillary studies is particularly useful in these cases.

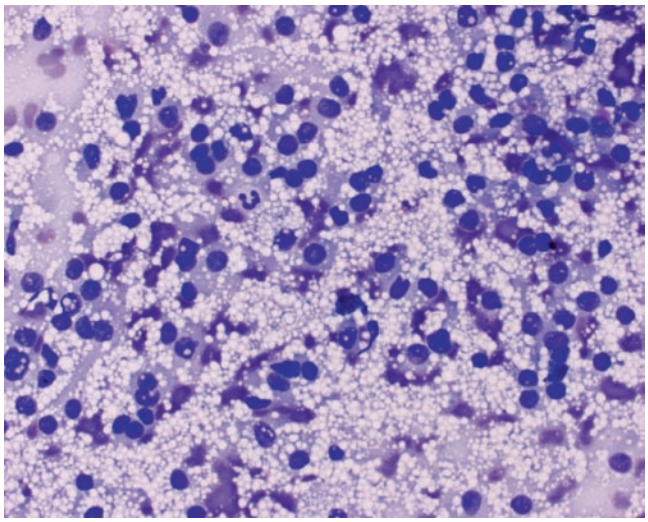

Fig. 23.15 Adrenal cortical lesion. Aspirates from cortical lesions, both hyperplasia and neoplasms, are indistinct from aspirates of normal adrenal cortex. The smears are cellular with adrenal cortical cells dispersed singly; the cytoplasm detaches easily during smear preparation and due to lipid content lends a microvesicular appearance to the background. Focal anisonucleosis ("endocrine atypia") has no significance. Diff-Quik® stain, ×400

Occasionally, an unsuspected case of myelolipoma can be sampled by FNAB. At low power, the aspirated material appears cellular with adipose tissue in the background. At higher magnification, immature myeloid precursors (myelocytes and metamyelocytes), erythroid precursors, and megakaryocytes are identified, almost mimicking a bone marrow aspirate [85].

Pheochromocytomas are rarely aspirated, as they are, most of the time, suspected from the clinical presentation and laboratory hormonal work-up. However, there are situations when pheochromocytomas are nonfunctional and therefore, not detected by the hormonal studies or a patient with known malignancy evaluated for adrenal metastases has no hormonal studies performed. In such cases, FNAB may target and sample this unsuspected pheochromocytoma. Aspirate smears are hypercellular with the neoplastic cells arranged singly or in loose clusters and numerous stripped nuclei in the background. The majority of the cells are epithelioid, oval or polygonal, with abundant delicate cytoplasm containing fine neurosecretory granules (red on Diff-Quik®), and indistinct cell borders (Fig. 23.16a). A small proportion of cells are spindle-shaped. The nuclei demonstrate considerable anisonucleosis, with some enlarged, bizarre nuclei with prominent nucleoli and other smaller nuclei with

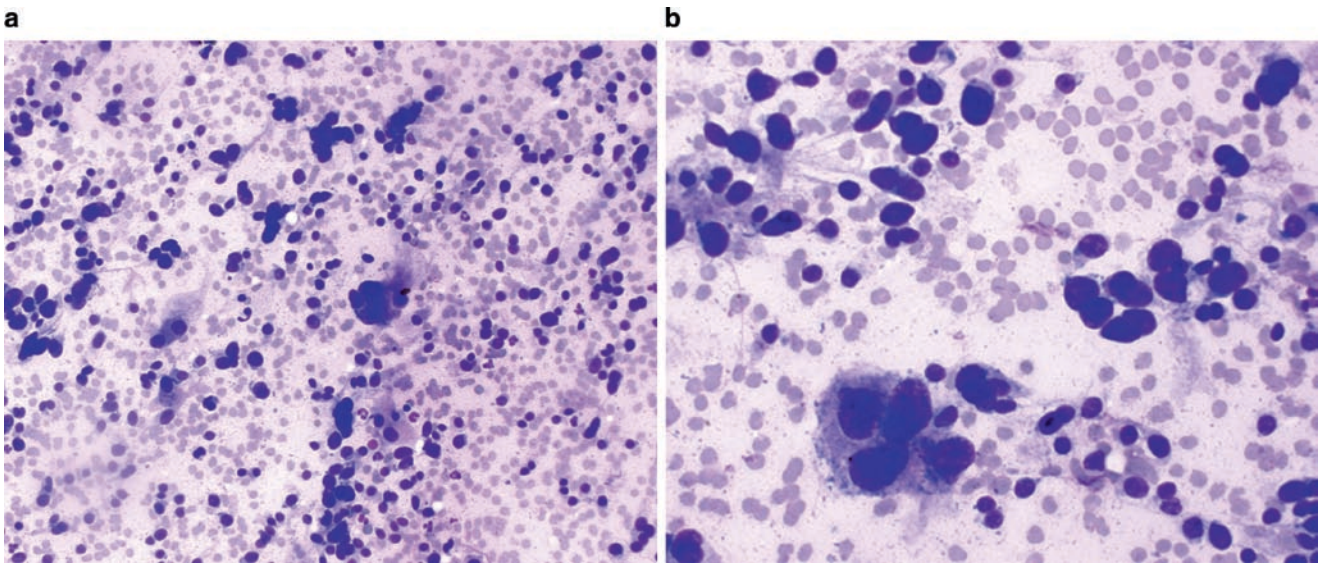

Fig. 23.16 (**a**) Pheochromocytoma. Aspirates are usually cellular with the neoplastic cells arranged predominantly singly. There is great morphologic variability among the tumor cells: small, round-oval cells, spindle-shaped cells, and large epithelioid cells with bizarre nuclei. Note the delicate cytoplasm with fine red granules in some of the tumor cells. Diff-Quik® stain, ×200. (**b**) Pheochromocytoma. Moderate to large neoplastic cells with abundant cytoplasm and large, bizarre nuclei with granular chromatin. Diff-Quik® stain, ×400

conspicuous salt and pepper chromatin (Fig. 23.16b). Intranuclear cytoplasmic inclusions may be seen occasionally [77, 86, 92, 93]. Despite obvious nuclear atypia, mitotic figures are infrequent. Pheochromocytoma can be *confused* with a metastatic carcinoma and sometimes it is difficult to differentiate it from an adrenal cortical carcinoma on cytomorphology alone. Obtaining a cell block for immunohistochemical stains is important, as pheochromocytoma is positive for synaptophysin and chromogranin, and negative for alpha-inhibin and melan-A [86].

Neuroblastoma is the fourth most common malignancy of childhood. It is a primitive neoplasm of neuroectodermal origin that arises from the adrenal gland and other sites containing sympathetic nervous system. Aspirates of neuroblastomas are hypercellular, composed of a uniform population of cells with high nuclear to cytoplasmic ratio distributed as scattered, single cells or cohesive groups with nuclear molding [94]. Nuclei are round, oval, with uniformly distributed fine chromatin and inconspicuous or absent nucleoli (Fig. 23.17a). The background shows necrotic debris and apoptotic bodies. Most helpful morphologic features that can provide a clue to the diagnosis, although not always seen are the presence of neuropil and Homer Wright rosettes. Neuropil can be seen in both Diff-Quik® and Papanicolaou stains, and appears as a fibrillary background matrix. Homer Wright rosettes consist of neuroblasts arranged around neuropil (Fig. 23.17b). The differential

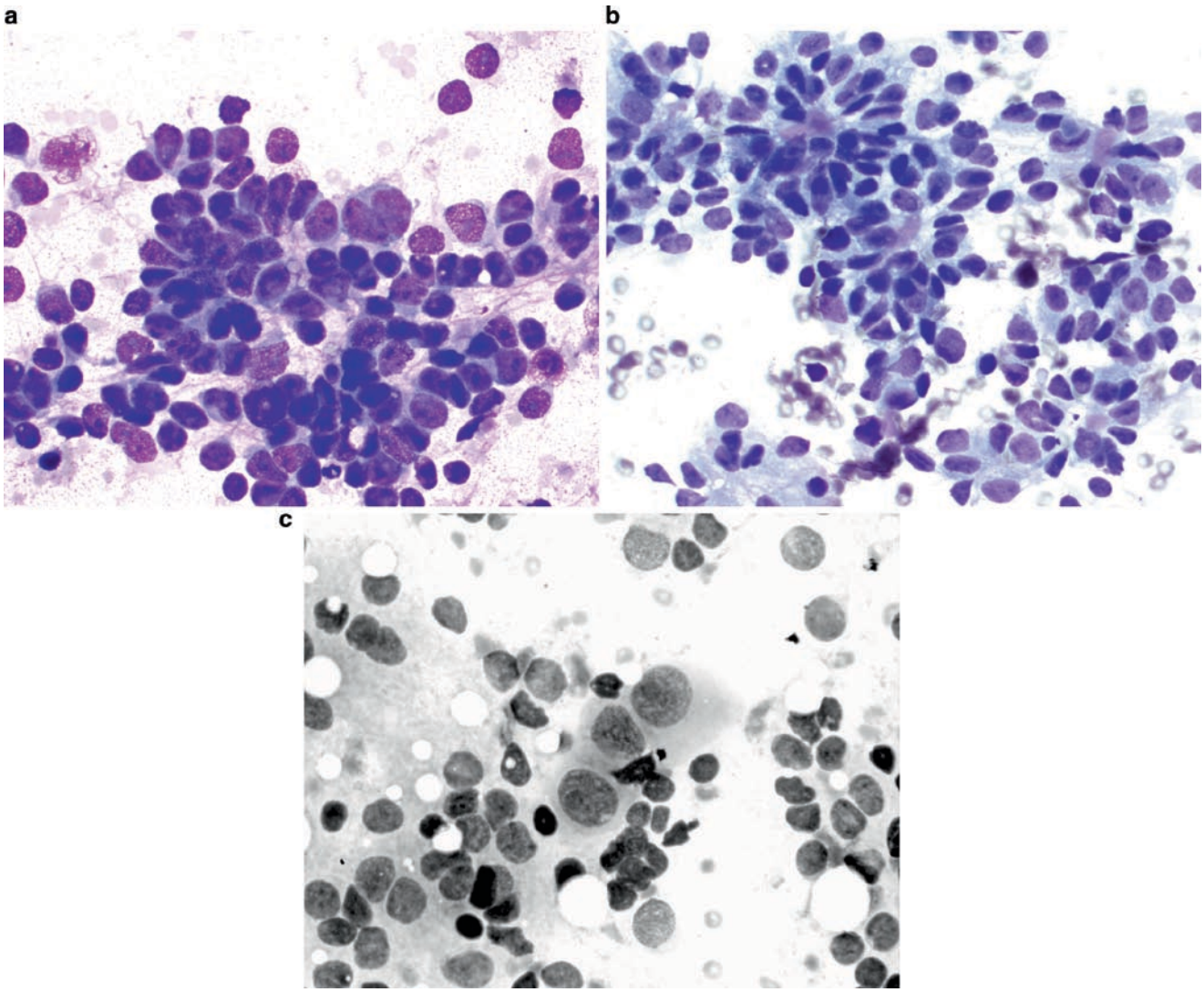

Fig. 23.17 (**a**) Neuroblastoma. Aspirates show the features of a small round blue cell tumor of childhood, specifically: uniform cells with high nuclear to cytoplasmic ratio, round-oval uniform nuclei with finely granular chromatin. Apoptotic bodies are seen in the background. Diff-Quik® stain, ×400. (**b**) Neuroblastoma. The presence of Homer Wright rosettes is a helpful morphologic feature in neuroblastoma. The rosettes consist of tumor cells arranged around a pink, fibrillary matrix which represents neuropil. Diff-Quik® stain, ×400. (**c**) 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 nucleoli. Papanicolaou stain, ×400

diagnosis includes other pediatric small round blue cell tumors, such as: Wilm's tumor, primitive neuroectodermal tumor (PNET)/Ewing's sarcoma, rhabdomyosarcoma, and malignant lymphoma. Immunohistochemical stains performed on the cell block reveal tumor cells positive for neuroendocrine markers (neuron-specific enolase (NSE), synaptophysin, chromogranin, CD56) and neurofilament proteins. Additional ancillary tests, for DNA ploidy analysis, N-myc gene amplification, and 1p deletion help determine prognosis and predicting response to therapy. Multiple methodologies are available for detecting N-myc gene amplification, including interphase fluorescent in situ hybridization on cytologic material [95]. Differentiated lesions (ganglioneuroblastoma) will have Schwannian-type spindle cells and ganglion-like cells (binucleated or multinucleated cells with prominent nucleoli and moderate amount of granular cytoplasm) [96] (Fig. 23.17c). Ganglioneuromas have a mixed population of mature ganglion cells, spindle cells, matrix of Schwannian origin, and collagen. As the diagnosis of ganglioneuroma relies on a lack of neuroblasts, and requires adequate sampling, as such it is usually suggested but not definitively diagnosed on aspirate smears.

Metastatic disease is one of the most frequent diagnoses encountered in aspiration of adrenal glands. Metastases to the adrenal glands from small cell and non-small cell lung cancer, colonic adenocarcinoma, breast carcinoma, renal cell carcinoma, malignant melanoma have been reported [77, 82–84]. Distinguishing primary from metastatic disease is usually straightforward, especially if the primary tumor is known and histology or cytology is available for review. There are, however, several difficult areas: (1) to differentiate an adrenal cortical carcinoma from some other metastatic lesions, in particular renal cell carcinoma, based on cytomorphology alone; (2) to misinterpret the abundant stripped nuclei of normal adrenal cortex or benign cortical lesions as small cell carcinoma. The presence of nuclear molding and individual cell necrosis and lack of microvesicular background support a diagnosis of metastatic small cell carcinoma. Alpha-inhibin and melan-A (A103) immunohistochemical stains performed on cell block are useful in identifying adrenocortical lesions (both benign lesions and adrenal cortical carcinomas) with a diffuse cytoplasmic granular staining pattern, and they are not expressed in renal cell carcinoma [97–99].

23.5 Summary

FNAB is a safe, accurate, economical, and *minimally* invasive technique for sampling of endocrine organs. Characteristic well-established cytologic criteria allow specific diagnosis of the majority of endocrine lesions. FNAB is widely used for sampling of both palpable and non-palpable thyroid nodules. Image-guided FNAB allows for sampling of parathyroid and adrenal glands, preferably with a cytopathologist on-site for preliminary interpretation. Good technical skills and adequate experience in obtaining and preparing samples are critical to the overall effectiveness of this procedure. With adequate knowledge of the cytomorphology, supplemented by appropriate ancillary techniques, many diagnoses can be made *without* resorting to surgical intervention. The specific information obtained from the FNAB specimen is used to triage patients, and thereby avoid invasive, costly, and unnecessary surgical procedures.

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Chapter 24
Multiple Endocrine Neoplasia Syndrome

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24.1 Discovery of MEN Syndromes

Multiple endocrine neoplasia syndrome type 1(MEN 1) and 2(MEN 2) are hereditary endocrine tumor syndromes characterized by multiple tumors in multiple organs. These syndromes, for which the responsible genes are *MEN1* for MEN 1 syndrome and *RET* for MEN 2 syndrome, are transmitted in an autosomal dominant pattern. In 1954, MEN 1 syndrome was first recognized by Wermer who reported a group of patients with adenomas of different endocrine organs and noted that the disease was heritable in an autosomal dominant pattern (1). This was followed by a single autopsy case report of a patient with bilateral thyroid carcinoma, bilateral pheochromocytoma, and a parathyroid adenoma by Sipple, and this combination of tumors was pointed out to be more than coincidental (2). These syndromes were designated as multiple endocrine adenomatosis type 1 and type 2 by Steiner et al. and later revised as multiple endocrine neoplasia syndromes, because some of the tumors were malignant (3). The MEN syndromes attracted great interests among oncologists, endocrinologists, surgeons, urologists, pathologists, and other researchers, because MEN 2 proved to be a genetic cancer syndrome whose responsible gene (*RET*) was elucidated (4), enabling family screening and early detection of asymptomatic patients by examining germline mutations of the *RET* protooncogene. This progress opened new unexplored fields of research, and more than 5,000 publications were identified with keywords of "multiple endocrine neoplasia syndrome" in a PubMed literature survey on December 1, 2008. Several other genetic cancer syndromes involving multiple endocrine organs, such as Carney complex, von Hippel-Lindau syndrome, Cowden syndrome, hyperparathyroidism jaw tumor syndrome, etc. have been reported, but they are not designated as MEN type 3 or type 4. This chapter focuses on classic MEN syndromes, both MEN 1 and MEN 2.

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Histopathologic findings of tumors of MEN 1(Table 24.1) and MEN 2 (Table 24.2) syndromes are reviewed briefly in this chapter, because most are described in other chapters, and their histopathologic characteristics are essentially the same as sporadic tumors. This chapter emphasizes neuroendocrine carcinoma of the pancreas (NEC) in MEN 1 patients and medullary (C cell) carcinoma (MTC) of the thyroid in MEN 2 patients, because these two malignant tumors have more prognostic impact on MEN patients. This review also focuses on molecular genetics and tries to explain gene functions of *MEN1* and *RET*, altered cell functions and underlining genotype–phenotype correlations. The third point in this chapter explains the recent treatment strategy with evidences of patients' outcome.

24.2 Histopathologic Characteristics of Tumors in MEN 1 and MEN 2 Syndromes

24.2.1 Multiple Tumors and Bilateral Tumors in MEN Patients and Precursor Lesions

The histologic features of tumors in patients with MEN 1 or MEN 2 syndromes are identical to those of sporadic tumors, with the exception of the multiplicity and bilaterality of tumors. Tumors in MEN patients are often *multiple* in one organ, such as pituitary adenomas, parathyroid adenomas, and neuroendocrine tumors of the pancreas in MEN1 patients (Fig. 24.1), and *bilateral*, such as pheochromocytomas of adrenal medulla and MTCs in bilateral lobes of the thyroid (Fig. 24.2) in MEN 2 patients. A precursor lesion or early stage of MTC in patients with MEN 2 was found by Wolfe et al. and reported as C cell hyperplasia (Fig. 24.3) in 1973 (5) and that of NET of the pancreas in MEN1 as microadenomatosis (Fig. 24.4) by Kloppel in 1986 (6, 7). Microscopic C cell proliferation is not an example of intrathyroidal metastasis but noninvasive C cell neoplasia with intact basement membrane under ultrastructural observations by DeLellis et al. and Kakudo et al. (8, 9), and it was confirmed by

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Table 24.1 Neoplastic lesions of MEN1

Endocrine neoplasms	Others
1. Parathyroid hyperplasia/adenoma/carcinoma	1. Lipomas
2. Enteropancreatic tumors	2. Angiofibromas
Gastrinoma, insulinoma, nonfunctioning tumor	3. Collagenomas
3. Pituitary tumor	4. Pheochromo cytoma
Prolactinoma, GH-secreting tumor etc.	
4. Foregut carcinoid	
Thymic carcinoid, bronchial carcinoid	
Gastric carcinoid (ECLoma)	

Table 24.2 Neoplastic lesions of MEN 2

Endocrine neoplasms	Others
1. Parathyroid hyperplasia/adenoma/carcinoma	1. Ganglioneuromatosis
2. Thyrod C cell (medullary) carcinoma	2. Megacolon
3. Pheochromocytoma/medullary hyperplasia	3. Mucosal neuroma
	1. Marfanoid habitus
	2. Thickened corneal nerve
	3. Hirschspurung's disease
	4. Cutaneous lichen amyloidosis

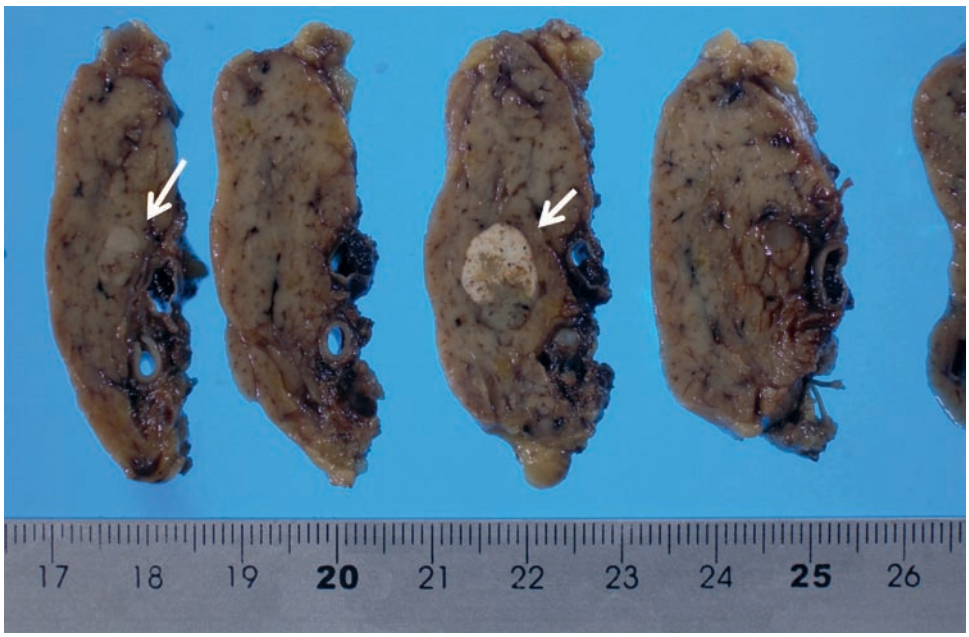

Fig. 24.1 Two macro-tumors (*white arrows*) are found in the pancreas from MEN 1 patient

demonstration of the basement membrane with immunohistochemistry by McDermott et al. (10) . This precursor lesion was regarded as carcinoma in situ (non-invasive early neoplastic lesion) rather than non-neoplastic, reactive or physiological proliferation (9, 11, 12). This C cell lesion explains the multiplicity of MTC and that tumors often grow in the contralateral lobe when remnant thyroid tissue is left after non-total thyroidectomy. It is important to note that this tumor is mostly not recurrence or metastasis to the remnant thyroid tissue, but is a *de novo* tumor in the thyroid from the precursor lesion. This explains why MTC patients with MEN 2A syndrome have a better outcome even if they have multiple tumors in bilateral lobes than sporadic MTC patients with a single primary tumor. Adenoma/hyperplasia of the pituitary, adenoma/hyperplasia of the parathyroid glands, carcinoid of the thymus, lungs and alimentary tract, and microadenomatosis/NET of the duodenum and endocrine pancreas in MEN 1 patients are reported as multiple, and adenoma/hyperplasia of the parathyroid glands, C cell hyperplasia/carcinoma of the thyroid gland, ganglioneuroma of the alimentary tract, and hyperplasia/pheochromocytomas of the adrenal medulla in patients with MEN 2 are multiple, bilateral, and diffusely observed. These multiple or bilateral tumors are assumed to occur from precursor lesions, which are observed as bilateral and often multiple in these organs. The distinction between the precursor lesion and eventual tumor is not well established, and size-based histopathologic criteria may not have been confirmed, because precursor lesions carry the same genetic abnormality even in the non-invasive stage (7).

The majority, but not all, of these precursor lesions occur in the context of genetic cancer syndromes. It has been suggested that these multiple microadenomatoses of the endocrine pancreas are a hallmark of MEN 1 and multicentric C cell

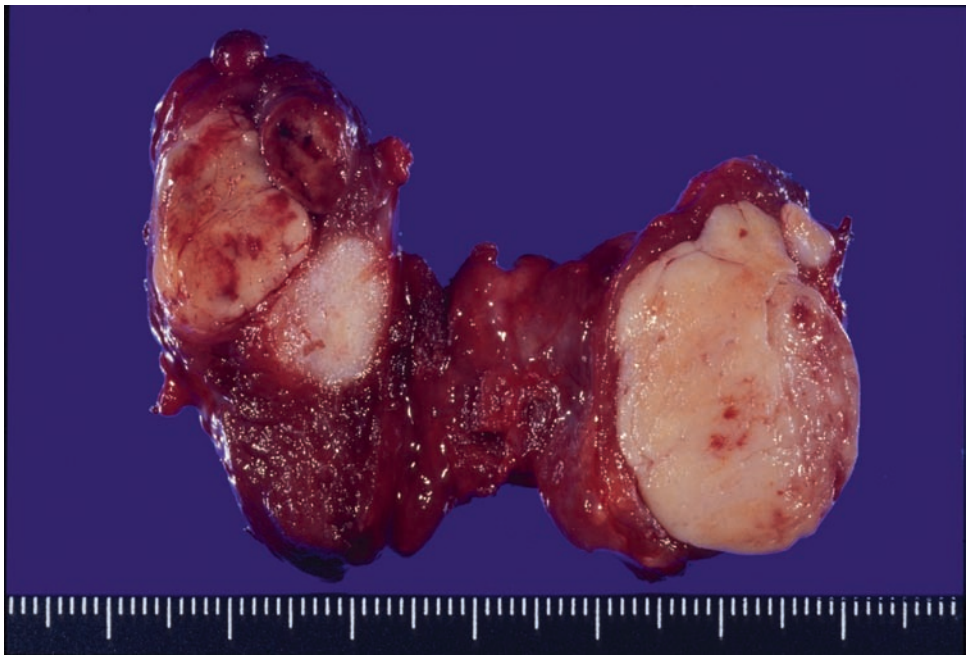

Fig. 24.2 Bilateral medullary carcinomas are found in right and left lobes of the thyroid gland from MEN 2B patients. The cut surface of MTC is ivory white and well demarcated from the thyroid parenchyma (mahogany brown)

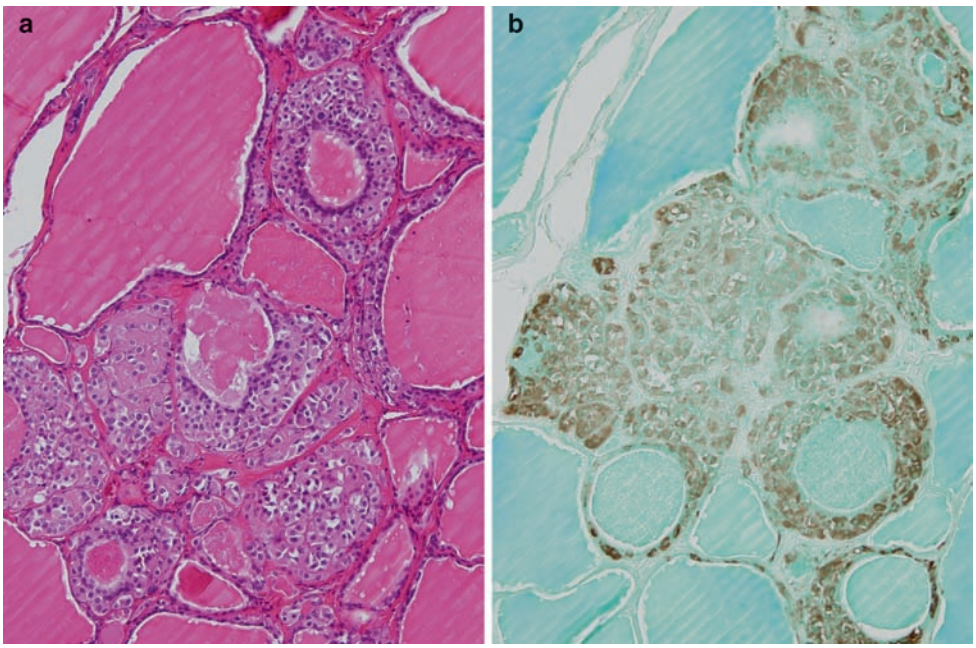

Fig. 24.3 **a** C cell hyperplasia and nodular hyperplasia or microscopic C cell tumor are found in the thyroid parenchyma from MEN 2A patient. **b** Their C cell nature is confirmed with immunohistochemistry for calcitonin. They are seen as rings, surrounding colloid follicles, or small nodular growth, replacing follicles.

hyperplasia of the thyroid is of MEN 2. These precursor lesions and multiplicity in the affected organs are common findings in genetic cancer syndromes, and these multiple precursor lesions may link MEN 1, MEN 2, and the other genetic cancer syndromes such as, Cowden syndrome, Carney complex and familial adenomatous polyposis syndrome, etc.

It is of note that C cell hyperplasia in the thyroid gland and hyperplasia of the endocrine pancreas have been reported

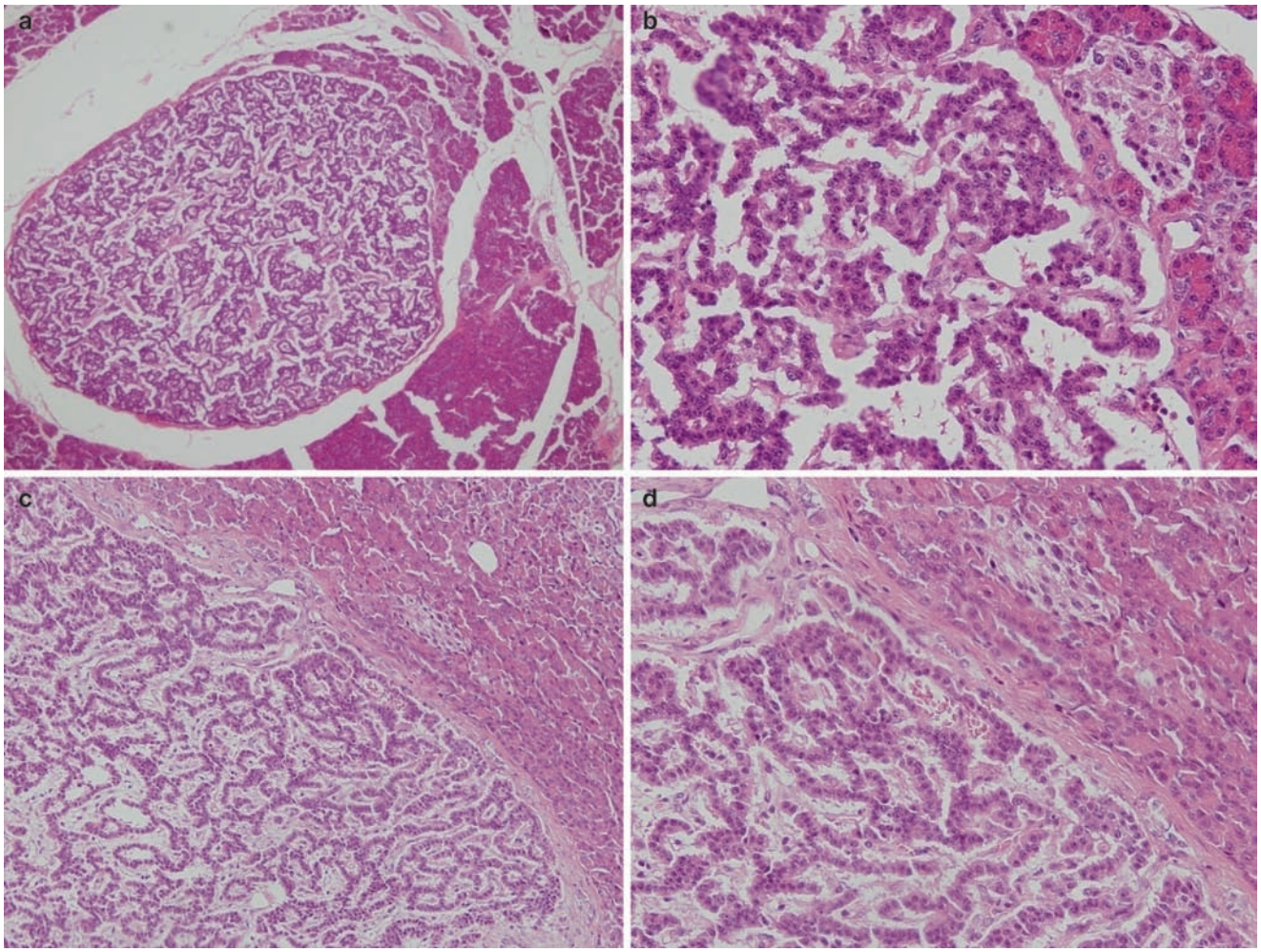

Fig. 24.4 **a** A small micro-adenoma, found in the pancreas from a MEN 1 patient. A higher magnification is shown in **b** and a ribbon and trabecular growth without capsule is noted in the micro-adenoma. A second adenoma with capsule from the same patient is shown in **c** and a higher magnification is shown in **d**

in non-MEN patients and sometimes in normal individuals. Guyetant et al. reported C cell clusters, which fulfilled C cell hyperplasia criteria in at least three fields at ×100 magnification containing more than 50 C cells, in 14 out of 42 normal autopsy thyroid glands (13)

24.3 Pathology of MEN 1

24.3.1 Pituitary Gland

The prevalence of pituitary tumors in MEN 1 patients varies between 10 and 50%, but the prevalence of MEN 1 pituitary tumors was as low as 0.6% in sporadic tumors (14–16). The pituitary adenomas in MEN 1 and non-MEN 1 patients were identical (14). A recent study by Trouillas et al. in 77 French patients of pituitary lesions in MEN 1 syndrome clarified that MEN 1 pituitary tumors were significantly larger, more often invasive, and had a lower response to treatment than non-MEN 1. Multiple adenomas were observed in 4% and significantly, more frequently observed in MEN 1 than in non-MEN patients. Folliculostellate cells (immunoreactive for S-100), were slightly more frequent in MEN 1 tumors (20.6% vs. 7.9%) (16). Capella et al. reviewed pituitary glands of MEN 1 patients and stated that adenomas in MEN 1 were more often functioning, more often involved growth hormone (GH) or were prolactin (PRL) producing, and were more frequently plurihormonal when compared with sporadic tumors in the general population (17). However, in a series of Trouillas's patients, the frequency of functional pituitary adenomas was identical in the two groups (72% MEN 1 vs. 64% non-MEN 1), but the frequency of plurihormonal adenoma

had a significantly higher incidence in the MEN 1 group (16, 18). In autopsy cases of MEN 1 patients, Capella et al. identified that PRL adenoma or mixed GH-PRL cell adenomas were multiple and were associated with PRL or GH cell hyperplasia of the peritumoral parenchyma of anterior pituitary (17).

It is of note that in one of the two patients with acromegaly, it was related to a pancreatic tumor from which GH-releasing hormone had been isolated, rather than primary GH adenoma of the pituitary (16).

24.3.2 Parathyroid Gland

Hyperparathyroidism is the most common manifestation (up to 80%) of MEN 1 patients and histopathologic examination of the parathyroid often reveals hyperplasia or adenoma and rarely carcinoma in multiple parathyroid glands (19–21). Parathyroid hyperplasia is characterized by an increased parenchymal cell mass and stromal fat cells are decreased. Parathyroid glands from MEN patients show chief cell hyperplasia of the diffuse and nodular type. Chief cell proliferation predominates in most cases, but a mixture of oncocytes and chief cells is common finding (Fig. 24.5). This genetic lesion is identical to secondary hyperparathyroidism, which also involves multiple parathyroid glands (19–21). In MEN 1 patients, multiple adenomas occur in a background of polyclonal hyperplasia (19–21).

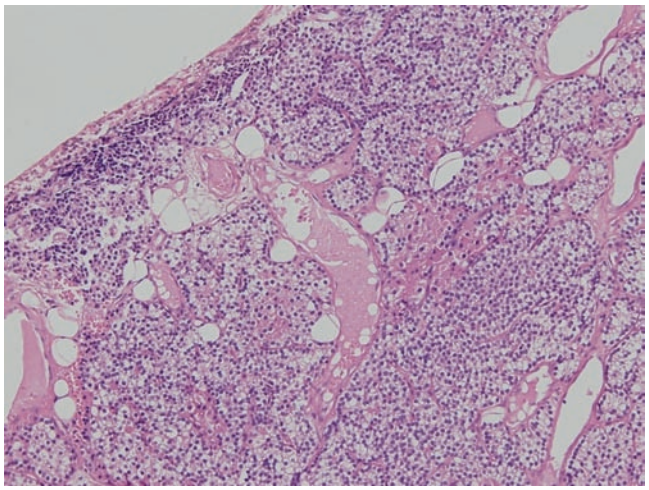

Fig. 24.5 Diffuse hyperplasia of parathyroid gland found in MEN 1 patient. Decreased fat cells and increased parenchymal tissue composed of chief cells with small clusters of oncocytes are seen

24.3.3 Neuroendocrine Tumors (NETs) and Precursor Lesions of Duodenum and Pancreas

The presence of multiple small endocrine cell nodules and micro-tumors (up to 5 mm) (Fig. 24.4) in the pancreas and duodenum has been referred to as microadenomatosis and is a hallmark of MEN 1(7, 22); however, microadenomatosis of the endocrine pancreas was observed in patients without MEN 1 syndrome, such as, without extrapancreatic manifestation or a family history of MEN 1, and Anlauf stated that the two conditions, one showing glucagon-producing tumors and the other displaying multiple insulinomas *might* represent new genetic tumor entities (7, 23). On the other hand, the microadenomatosis of the endocrine pancreas is reportedly rare in some genetic cancer syndromes, such as, von Hippel-Lindau syndrome (23).

Morphologically, NETs of duodenum and pancreas are well-differentiated tumors showing various histological patterns, characterized by ribbon, trabecular, solid, glandular, pseudorostte or tubular arrangements. The tumor cells are relatively uniform with wide, finely granular eosinophilic cytoplasm and a small, round to oval nucleus (Fig. 24.4). Immunohistochemical identification of hormonal markers, such as, insulin, glucagon, pancreatic polypeptides, gastrin, somatostatin and vasoactive intestinal polypeptide, is helpful to characterize tumor cell types and their specific hormonal products. However, immunohistochemical intensity or the number of positive cells is *not* related to the hormone symptoms. Sometimes, immunoreactive hormones can be localized to cells of non-functioning tumors and many tumors are composed of more than one phenotype (plurihormonal). Tumors containing pancreatic polypeptide and glucagon are most often identified, whereas tumors expressing insulin are less common and tumors containing gastrin or somatostatin are rare in the pancreas (22–31).

Classification of hyperplasia/microadenoma/macroadenoma of endocrine pancreas may be size-based and distinction between large hyperplastic islets and MEN-related microadenoma less than 1 mm may be practically difficult. In addition to its difficulty, clear-cut distinction between benign macroadenomas and neuroendocrine carcinomas (NECs) is not always possible and they are divided in three practical categories in new WHO classification, (1) well-differentiated endocrine tumor, benign behavior, and uncertain behavior, (2) well-differentiated endocrine carcinoma, functioning and non-functioning, and (3) poorly differentiated pancreatic endocrine carcinoma, small cell carcinoma and mixed exocrine–endocrine carcinoma (22). Heits et al. stated that tumors with a diameter of more than 2 cm have an increased risk of malignant behavior and those over 3 cm are

usually malignant (22). The most reliable evidence of malignancy is identification of metastasis to the regional lymph nodes or other organs or gross infiltration of adjacent organs. No stage classification has been applied to NECs of the pancreas. The NECs of pancreas are farther divided into well-differentiated endocrine carcinoma and poorly differentiated endocrine carcinoma, rare tumors with high mitotic count more than 10 mitoses/10 high power fields (HPFs), in WHO classification (22). The well-differentiated endocrine tumor exhibits a spectrum of biologic behavior, and a rare aggressive form is found in benign macroadenoma, borderline group, and well-differentiated endocrine carcinoma group, and Hochwald et al. proposed intermediate-grade group of carcinoma (24). This intermediate-grade of NEC is defined based on mitotic rate (more than 2 mitoses/50 high power fields) and presence of necrosis, and is reported to correlate with survival (24). Adverse prognostic factors of well-differentiated pancreatic endocrine tumors are, metastasis, gross invasion to the adjacent organs, tumor diameter larger than 2 cm, angio invasion, perineural invasion, mitoses more than 2 per 10 HPF, Ki-67/MIB-1 labeling index more than 2% and necrosis (22) (Fig. 24.6).

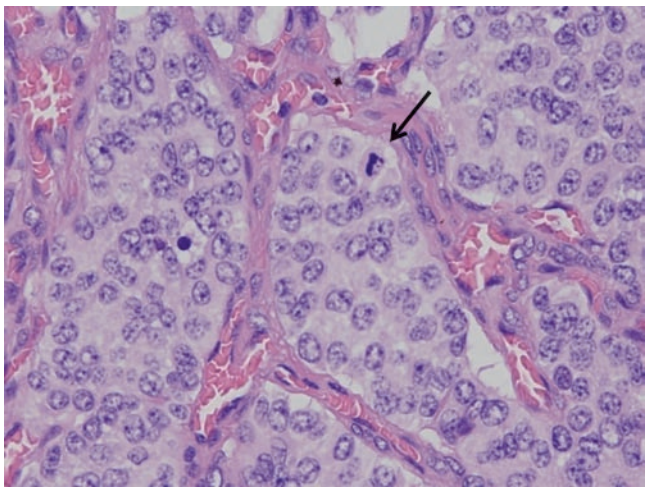

Fig. 24.6 A mitotic figure (*arrow*) in intermediate-grade group of well-differentiated endocrine carcinoma of pancreas in MEN 1 patient. Note higher nuclear grade and smaller N/C ratio of the tumor.

24.3.4 Other Endocrine Manifestations and Non-endocrine Tumors

MEN1 causes combinations of more than 20 endocrine and non-endocrine tumors (Table 24.1) (24–31). A wide range of other tumors occurs in MEN 1, including thymic and bronchial neuroendocrine tumors (carcinoids), cortical adenoma of the adrenal glands, follicular tumors of the thyroid glands and non-endocrine tumors, such as meningeomas, ependymomas, lipomas and facial angiofibromas etc (14, 25–31).

24.4 Genetics

24.4.1 Structure and Function of the *MEN1* Gene

A genetic mapping study using DNA from MEN 1 patients indicated that the *MEN1* gene is located at chromosome 11q13 (32). In 1997, the *MEN1* gene was isolated, which consisted of 10 exons distributed over 9 kilobases (kb) (Fig. 24.7a). It is transcribed as a 2.8-kb mRNA and encodes a protein of 610 amino acids (67 kDa), referred to as menin

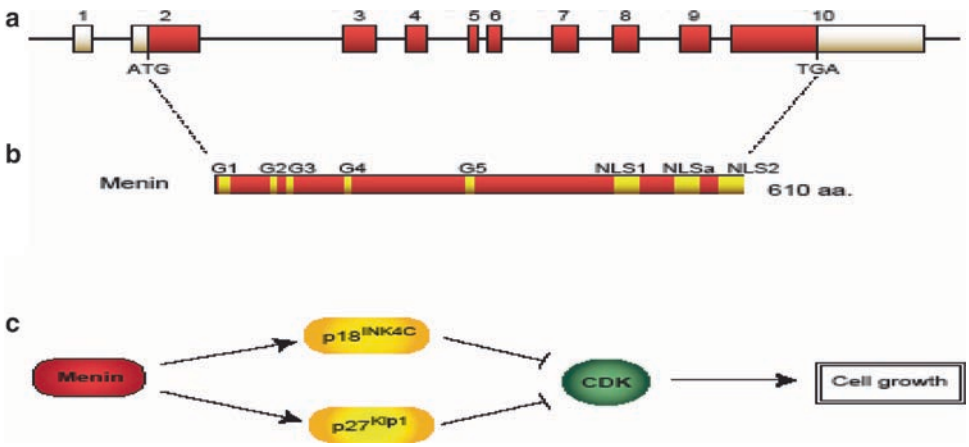

Fig. 24.7 Structural organization of the human *MEN1* gene, its product (Menin) and function. **a** The human *MEN1* gene consists of 10 exons and the coding region is indicated by red. **b** The *MEN1* gene encodes a 610 amino acid protein referred to as menin. Menin has three nuclear localization signals at codons 479–497 (NLS1), 546–572 (NLSa) and 588–608 (NLS2), and five putative guanosine triphosphatase sites (G1–G5) indicated by yellow. (**c**) Hypothetical tumor suppressor function of menin

(33, 34), which is ubiquitously expressed and located primarily in the nucleus (35). Menin has, at least, three nuclear localization signals (NLSs) in the carboxy-terminal region (36) (Fig. 24.7b). Menin homologs have been identified in various species including mouse, rat, zebrafish, fruit fly, and snail, but not in the yeast *Saccharomyces cerevisiae* and nematode *Caenohabditis elegans* (37–44).

Menin has no homology to any known proteins or sequence motifs and has been shown to interact with the activating protein-1 (AP-1) transcription factor JunD (45), nuclear factor-kappa B (NF-kappaB) family members (46), Smad family members (Smad3 and Smad1/5) (47), mouse placental embryonic (Pem) expression gene protein (48), nm23 (49) and Runx2 (50). Studies revealed that these protein–protein interactions are involved in various cellular processes, including transcriptional regulation, genome stability, cell division and proliferation; for example, JunD and NF-kappaB-mediated transcriptional activity is suppressed by menin, whereas Smad 3-mediated transcription appears to be enhanced. Inactivation of *MEN1* increases proliferation and causes a transition from the G_0/G_1 to S phase in pancreatic islet cells (51). In addition, although direct binding of menin to DNA has not been clearly demonstrated, several target genes positively regulated by menin have been reported (52–54). These include *HOXA9* and cyclin-dependent kinase inhibitors, $p18^{Inc4c}$ and $p27^{Kip1}$, suggesting that menin functions as a tumor suppressor by regulating p18 and p27 expressions (Fig. 24.7c).

24.4.2 Mutations in the MEN1 Gene

A recent database (the NCBI PubMed literature database) search included 1,336 *MEN1* gene mutations (1,133 germline mutations and 203 somatic mutations), which consisted of 459 different germline mutations and 167 different somatic mutations (55). Among these, 61 were detected in both germline and somatic mutations, yielding 565 different *MEN1* mutations.

Various mutations, including single nucleotide alterations, frameshift insertions or deletions, in-frame insertions or deletions, and large deletions, have been identified, which consisted of 41% frameshift insertions or deletions, 23% nonsense mutations, 20% missense mutations, 9% splice site mutations, 6% in-frame insertions or deletions and 1% large deletions (55). Frameshift mutations and nonsense mutations produce truncated proteins, thus supporting the function of menin as a tumor suppressor.

These mutations were scattered throughout the whole *MEN1* gene and no mutation hot spot has been identified; however, some mutations (so-called warm spots) have been encountered in unrelated families (55). Mutations at four sites accounted for 12.3% of all mutations (c.249_252delGTCT, deletion at codons 83-84; c.1546_1547insC, insertion at codon 516; c.1378C>T (Arg460 Stop); and c.628_631delACAG, deletion at codons 210-211) (Table 24.3) (56). Three mutations are insertions or deletions in repetitive sequences, suggesting that they occur by slippage-mediated mutations. In addition, five other mutations occurred in over 1.5% of affected families (Table 24.3) (55).

Table 24.3 MEN1 mutations detected in over 1.5% of affected families (from ref. [55])

Mutation	Exon	Codon	Type of mutation	Frequency (%)
c.249_252delGTCT	2	83–84	Frameshift mutation	4.5
c.292C > T	2	98	Arg98Stop	1.5
c.358_360delAAG	2	120	In-frame mutation	1.7
c.628_631delACAG	3	210–211	Frameshift mutation	2.5
c.784-9G > A	Intron 4	–	Splice-site mutation	1.9
c.1243C > T	9	415	Arg415Stop	1.5
c.1378C > T	10	460	Arg460Stop	2.6
c.1546delC	10	516	Frameshift mutation	1.8
c.1546_1547insC	10	516	Frameshift mutation	2.7

Five-to-ten percent of MEN1 patients do not appear to carry *MEN1* mutations in the coding sequence or adjacent splicing sites. In these cases, it is necessary to investigate mutations in the promoter–enhancer region or 5′ or 3′-untranslated region, although it may be difficult to determine whether these alterations represent real mutations, non-pathologic mutations or polymorphism.

Despite the widespread tissue expression of menin, tumor development in MEN 1 is confined to limited tissues. The mechanisms underlying this specificity remain *elusive*.

24.4.3 Genotype–Phenotype Correlation

No clear genotype–phenotype correlation in MEN 1 has been established. Neither the location of the mutation nor the mutation type appears to have any effect upon the phenotype. MEN1 families with the Burin or prolactinoma variant, which is characterized by a high occurrence of prolactinoma and a low occurrence of gastrinoma have been reported (57). Three distinct nonsense or frameshift mutations (Tyr312Stop, Arg460Stop and 1021delA) have been identified in these families (58, 59). In addition, splice-site mutation (c.446-3C>G) has been reported in a large MEN 1 family from Tasmania which is characterized by an absence of somatotrophinomas (60); however, these mutations do *not* appear to be different from other mutations identified in classical MEN 1.

In addition, *MEN1* mutations have been reported in 42 families with isolated hyperparathyroidism (FIHP). The incidence of missense mutations (38%) in these families was significantly higher than that in MEN1 families (20%) (53). These findings may suggest a possible association between missense mutations and the FIHP phenotype, although the mutations associated with FIHP are also scattered throughout the coding region, as observed in MEN 1 patients. Furthermore, the fact that the same mutations including protein-truncation mutations as those observed in MEN 1 patients have been identified in FIHP makes it difficult to establish a clear genotype–phenotype correlation.

24.4.4 MEN1 Mutations in Sporadic Endocrine Tumors

Somatic *MEN1* mutations have been investigated in sporadic cases of parathyroid, pancreatic islet and anterior pituitary tumors, because LOH involving chromosome 11q13 has also been observed in 5–50% of these sporadic tumors. As a consequence, somatic *MEN1* mutations have been detected in ~20% of sporadic parathyroid tumors, ~40% of gastrinomas, ~15% of insulinomas, ~60% of glucagonomas, ~15% of non-functioning pancreatic tumors, and <5% of anterior pituitary adenomas (55, 61). In addition, somatic *MEN1* mutations have been detected in 10–35% of carcinoid tumors, angiofibroma and lipomas. Of these mutations, 40% are frameshift mutations and 18% are nonsense mutations, indicating that loss of menin function is involved in the development of some cases of sporadic endocrine tumors.

24.4.5 DNA Test for MEN 1

Since identification of the *MEN1* gene in 1997, DNA analysis has provided useful information for the clinical management of MEN 1 patients. Because MEN 1 is inherited in an autosomal-dominant fashion, it is important to identify normal or mutant gene carriers in family members. DNA test will *release* non-mutation carriers from further unnecessary clinical investigations and fear of disease. Earlier and more frequent biochemical and radiological screening for MEN 1 tumors will be undertaken in mutation carriers and appropriate intervention, including surgical treatment may be considered. However, the widely scattered locations of mutations in the *MEN1* gene make the DNA test time-consuming and expensive, and a positive result does not necessarily implicate intervention to prevent malignancy.

24.4.6 Clinical Aspects: MEN 1

MEN 1 has a high degree of penetrance, and the prevalence of MEN 1 patients showing clinical evidence was 43% at the age of 20 years, 85% at the age of 36 years, and 94% at the age of 50 years (62). It is desirable to detect MEN 1 index patients in an early phase because MEN 1-related tumors such as gastrinoma and thymic carcinoid are often malignant and are a major cause of death for MEN 1 patients (63, 64). To date, no phenotype–genotype correlation has been detected, but the most common clinical manifestation of MEN 1 is primary hyperparathyroidism. Therefore, MEN 1 screening for patients with hyperparathyroidism is important to detect index patients with MEN 1.

24.4.7 Clinical Aspects: Hyperparathyroidism in MEN 1

Although MEN 1 accounts for only 2–4% of all cases of hyperparathyroidism (65), it is impossible to screen all patients with hyperparathyroidism for *MEN1* mutation. On the other hand, primary hyperparathyroidism had nearly 100% penetrance by age 50 years (26–30). The typical age group of onset is 20–25 years, that is, 30 years earlier than from sporadic parathyroid adenoma (27–31). Brandi et al. and Hai et al. thus suggested that patients with hyperparathyroidism aged younger than 30 years are indicated for *MEN1* genetic analysis (66, 67). Our department recommends *MEN1* mutation analysis for patients with hyperparathyroidism younger than 30 years and has found *MEN1* mutation in 33% of these patients (Table 24.4). This incidence is quite high compared with the prevalence of MEN 1 in all hyperparathyroidism patients. Furthermore, patients with multiple enlarged parathyroid glands, co-existence or a past history of MEN1-related tumors, or a family history of hyperparathyroidism or MEN1-related tumors, are indicated for *MEN1* gene analysis (66). Although the incidence of patients indicated for genetic analysis decreases with increasing patient age (45% in 30s, 16% in 40s, 9% in 50s, and 3% in 60s in our

Table 24.4 Relationship between patient age and MEN1 mutation detected

Age (years)	Incidence of patients recommended gene analysis (%)	Incidence of MEN1 mutation detected in patients underwent gene analysis (%)
≤29	100	33
30–39	45	29
40–49	16	50
50–59	9	64
60≥	3	40

department), the incidence of MEN 1 patients older than 30 years in those indicated for analysis was high, 48%, in our department. Whether hyperparathyroidism patients have *MEN1* mutation is a very important issue because surgical procedures for patients with and without *MEN1* mutation are entirely different. Total parathyroidectomy with autotransplantation or three and a half parathyroidectomy is recommended for MEN 1 hyperparathyroidism (68–70), while parathyroidectomy of the enlarged glands is performed for patients with parathyroid adenoma without *MEN1* mutation. A flow chart of the strategies to differentiate MEN1 patients from those with hyperparathyroidism and the treatment for these patients is shown in Fig. 24.8.

Patients with MEN 1 hyperparathyroidism should be carefully followed to see whether other MEN 1-related tumors occur after parathyroidectomy. In our department, pancreatic tumor occurred in two patients during follow up. Needless to say, careful postoperative follow up is required also for patients indicated for *MEN1* genetic analysis but who have refused. Furthermore, patients who have undergone *MEN1* genetic analysis but who did not show *MEN1* mutation should also be constantly and carefully followed because genetic analysis failed to detect 10–20% of *MEN1* mutation (55, 71–73).

MEN 1 mutation can be detected in a high incidence of patients with primary hyperparathyroidism with co-existence or a past history of MEN1-related tumors; however, physicians can encounter such patients without *MEN1* mutation, designated as MEN 1 phenocopy (67, 74–79). Hai et al. demonstrated that 12 of 21 patients clinically diagnosed with MEN1 were negative for *MEN1* gene mutation and 11 of these patients had primary hyperparathyroidism (67). Four of these patients showed enlarged multiple parathyroid glands and no lesions other than hyperparathyroidism and GH-secreting pituitary tumor developing before 50 years of age. Sakurai et al. demonstrated that the average age of patients with MEN 1 phenocopy did not differ from that of familial MEN 1 patients (77). The incidence of phenocopy in patients with hyperparathyroidism and a past history of MEN 1-related tumors in our department was 50% and all patients had a single enlarged gland (parathyroid adenoma). How to treat and follow MEN 1 phenocopy remains controversial still, but regular biological and radiological tests could be necessary, for example, every year and 3 years, respectively, considering the possibility of missing *MEN1* mutation.

24.4.8 Clinical Aspects: Neuroendocrine Tumors of Pancreas in MEN 1

Neurondocrine tumors of pancreas in MEN 1 patients are detected in up to 80% of MEN 1 patients (27, 80, 81); they are usually multicentric and can occur in any lesion of the pancreas or the duodenum showing various biological

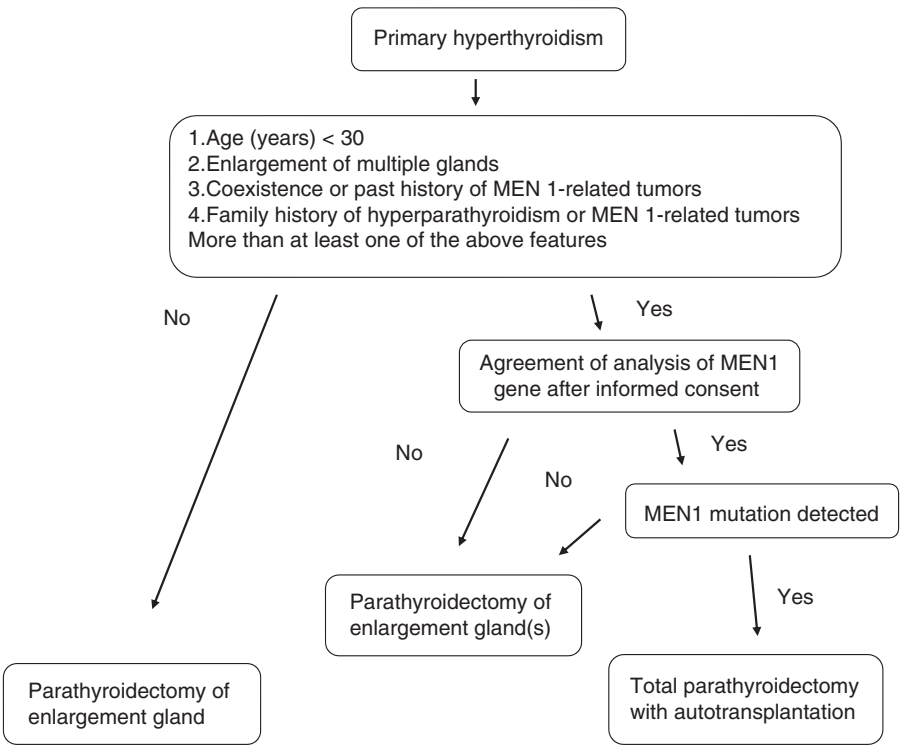

Fig. 24.8 Flow chart of extraction of patients having MEN1 mutation and their treatment strategies

aggressiveness from microadenomas to carcinomas with metastasis (82). Tumors express various hormones, often multiple hormones, such as combinations of glucagon, chromogranin, polypeptide, insulin, somatostatin, gastrin, VIP and serotonin (6, 82, 83). The prevalence of malignant tumor increased at over 30 years of age. In order to diagnose NETs of duodenum and pancreas, various hormonal analyses have been proposed. Fasting gastrin and gastric acid output, and fasting glucose and insulin are useful to diagnose gastrinoma and insulinoma, respectively (66, 83). These biological tests should be performed annually for patients suspected of being MEN 1 carriers. Imaging studies, such as computer tomography (CT) scan, magnetic resonance imaging (MRI) and somatostatin receptor scintigraphy (^{111}In-diethylenetriamine pentaacetic acid-octreotide scan [111In-DTPA octreotide scan]) are also useful to diagnose neuroendocrine tumors (84, 85); however, they are *not* much effective for gastrinoma and insulinoma.

Physicians should take special care when they encounter patients with Zollinger-Ellison syndrome due to gastrinoma. About 25% of patients with gastrinoma showed *MEN1* mutation and 40% of MEN 1 patients had gastrinoma. Furthermore, the prevalence of malignancy is high, with more than 30% morbidity (86–88). As indicated above, imaging studies are not very useful to detect gastrinoma (85); however, the recent techniques of endoscopic ultrasound, hepatic vein sampling for gastrin after arterial secretin injection and intraoperative endoscopy facilitate identification of its location (89). The indication of surgery for gastrinoma in MEN 1 patients remains controversial. Gastrinomas in MEN 1 patients are usually multiple and metastatic, and disease-free survival rates of gastrinoma in MEN 1 patients are much worse than those in patients without MEN 1 after surgery (87, 88, 90). Instead, proton pump inhibitors and H_2-receptor blockers are reported to be useful to control excess gastrin (91).

It is said that insulinoma can occur in MEN 1 patients at the age of 5 years. In contrast to gastrinoma, surgery is the first line of therapy of insulinoma when patients show hypoglycemia (66). One reason is that most insulinomas can be detected on intraoperative ultrasonography, even if they cannot be detected on preoperative imaging. For other excess hormone syndromes due to NETs, medical management is effective. Surgery can also be indicative, but no consensus is available as yet about the criteria for the indication of surgery.

It is *controversial* whether surgical treatment should be selected for asymptomatic NETs in MEN 1 patients. Some groups recommend surgery for cancer prevention, after biochemical diagnosis. Others advocate surgery when the tumor reaches 1 cm, and another group does not perform surgery unless the tumor exceeds 3 cm or tumor starts showing signs of developing (92–94).

24.4.9 Clinical Aspects: Pituitary Tumors in MEN 1

According to previous reports, the incidence of anterior pituitary adenoma in MEN 1 patients varies from 10 to 60% (27, 31, 95, 96). All types of pituitary adenoma except for gonadotropinoma have been reported (27, 76, 96). No significant relationships could be found between the type of *MEN1* gene mutation and the pathology of pituitary adenomas, that is, genotype–phenotype relationships are lacking. A multicenter study published in 2002 indicated that pituitary adenoma is observed in 42% of MEN 1 patients, which is larger and more aggressive than that in patients without MEN 1 (97). A more recent case-control study also showed that MEN 1 tumors are larger and histologically, more invasive than non-MEN 1 tumors (16). Furthermore, MEN 1 tumors are more frequently plurihormonal and it was concluded that *MEN1* genetic analysis is recommended for patients with associated PRL or GH-ACTH secretion in a tumor or multiple PRL and ACTH tumors. Brandi et al. recommended annual measurement of serum basal levels of PRL and IGF-1 and magnetic resonance imaging(MRI) as an imaging test every 3 years for likely MEN 1 carriers (66). For patients with abnormal biological tests, hypothalamic-pituitary testing is effective for evaluating pituitary lesions. Treatment for pituitary tumor in MEN 1 patients is the same as for non-MEN 1 patients, depending on the tumor characteristics.

24.4.10 Clinical Aspects: Other Tumors in MEN1 (Table 24.1)

Carcinoids of the thymus and the bronchium are also known as MEN 1-related tumors (98). Although patients remain asymptomatic until older age, these carcinoids are more aggressive than those occurring in non-MEN 1 patients. Carcinoid can also occur in the stomach; it is said to originate from type 2 gastric enterochromaffin-like cells (ECL cells) and is called ECLoma (99, 100).

Morphological changes in the adrenal cortex occurs in up to 40% of MEN 1 patients, and they are usually hyperplastic but non-functional (101–103). Various histologies from adenoma to carcinoma have been reported (102).

Subcutaneous or visceral lipomas have been reported in MEN 1 patients and their prevalences are up to 30% (27, 76, 104). They are multicentric and often induce cosmetic problems.

Facial angiofibroma and collagenoma are detected in up to 80% of MEN 1 patients and half of these patients had five or more lesions (105–107).

24.4.11 Screening of MEN1-Related Tumors

According to the consensus report by Brandi et al. (66), biochemical tests are recommended annually for parathyroid adenoma (ionized calcium and PTH) from 8 years old, for gastrinoma (gastrin, gastric acid output, secretin-stimulated gastrin) from 20 years old, for insulinoma (fasting glucose and insulin) from 5 years old, for other NETs (glucagon and proinsulin) from 20 years old, and for pituitary tumor (PRL and IGF-1) from 5 years old. Unfortunately, no appropriate biochemical tests are available for foregut carcinoid. Imaging studies, such as MRI for pituitary tumor, CT scan for foregut carcinoid, ^{111}In-DTPA octreotide scan, and MRI for enteropancreatic tumors are also recommended every 3 years.

24.5 Pathology of MEN 2

24.5.1 Parathyroid Glands

Parathyroid abnormalities are rather rare event in MEN 2 patients and the prevalence is approximately 20% (108). This event is most frequently observed in patients with having *RET* mutation in codon 634 and not in those with mutations in codon 883, 918 or 922. Histopathologic examination of the parathyroid often reveals diffuse or nodular hyperplasia, adenoma and rarely carcinoma in multiple parathyroid glands. This parathyroid lesion is identical to secondary (non-hereditary) hyperparathyroidism, which also involves multiple parathyroid glands. Carney et al. analyzed parathyroid lesions in MEN 2B patients and pointed out that parathyroid hyperplasia occurred rarely in MEN 2B patients, while it is found about 20% of patients in MEN 2A (19–21, 109).

24.5.2 Pheochromocytoma and Paraganglioma

MEN 2 syndrome is characterized by the development of bilateral and multicentric adrenal medullary tumors in addition to hyperparathyroidism and thyroid C cell carcinoma. In MEN 2A patients, approximately 50% of patients develop multiple and bilateral pheochromocytomas (110, 111). DeLellis et al. using morphometric analysis of the adrenal medulla from patients with MEN 2, at an early stage, showed a two- to threefold increase in volume and weight as compared to age- and sex-matched controls (111). The authors emphasized that the increase of total medullary mass resulted from diffuse and multifocal nodular proliferations of adrenal medullary cells within the glands and concluded that pheochromocytomas in patients with MEN 2 syndrome might represent an extreme degree of nodular hyperplasia of the medulla (111). The diagnosis of adrenal medullary hyperplasia is sometimes difficult and distinction between adrenal medullary hyperplasia and pheochromocytoma may be impossible.

Recent human genetic studies have now shown that 25–35% of patients have hereditary pheochromocytomas due to a germline mutation in one of the five pheochromocytoma susceptibility genes, *SDHB, SDHD, VHL, RET or NF1* gene (112–119). The loss of heterozygosity with *SDHB* mutations, but not with *RET* mutation, is correlated with malignant pheochromocytoma, and identification of germline mutations of *SDHB* gene in patients with pheochromocytomas is a high-risk factor for malignancy or recurrence (117–119).

24.5.3 C Cell (Medullary) Carcinoma of Thyroid (MTC)

Hazard et al. first identified MTC in 1959 as a distinct tumor entity separate from undifferentiated carcinoma of the thyroid because of its better prognosis. The authors pointed out its histopathologic characteristics; solid growth (non-follicular and non-papillary), amyloid stroma and a high incidence of lymph node metastasis (120, 121). He pointed out that this tumor had similarities to carcinoid tumors, suggesting neuroendocrine in nature (120). Williams identified the histogenesis of this carcinoma as C cell in 1966(122). Most of MEN 2 patients develop MTCs at a younger age than sporadic cases, and the average age at surgery was reportedly approximately 30–35 years for MEN 2A patients, 20–25 years for MEN 2B patients in comparison with 40–50 years for sporadic patients (120, 121, 123–135).

Franc et al. reported that 5-, 10-, and 15-year adjusted survival rates for 109 cases of MTCs with clinical symptoms (proband cases) were 80.7, 72.2, and 66.4%, respectively (131). These figures were in good agreement with previous publications, which included a significant proportion of early stage patients by family screening (125–130, 132, 133). Tumor metastases in surgical series of patients are frequent in the regional lymph nodes; however, hematogenous metastasis becomes more frequent in an autopsy series. Hematogenous metastases were found in the lungs (84.6%), liver (69.2%), bones (53.8%) in addition to the lymph nodes (100%) and body cavity (30.8%) in our 13 autopsy cases (136).

MTC in MEN 2 and familial MTC patients is usually bilateral and well demarcated but not capsulated (Fig. 24.2). It is a solid tumor with a cut surface of grey to pinkish or ivory white (Fig. 24.2). The tumor cells have a vesicular

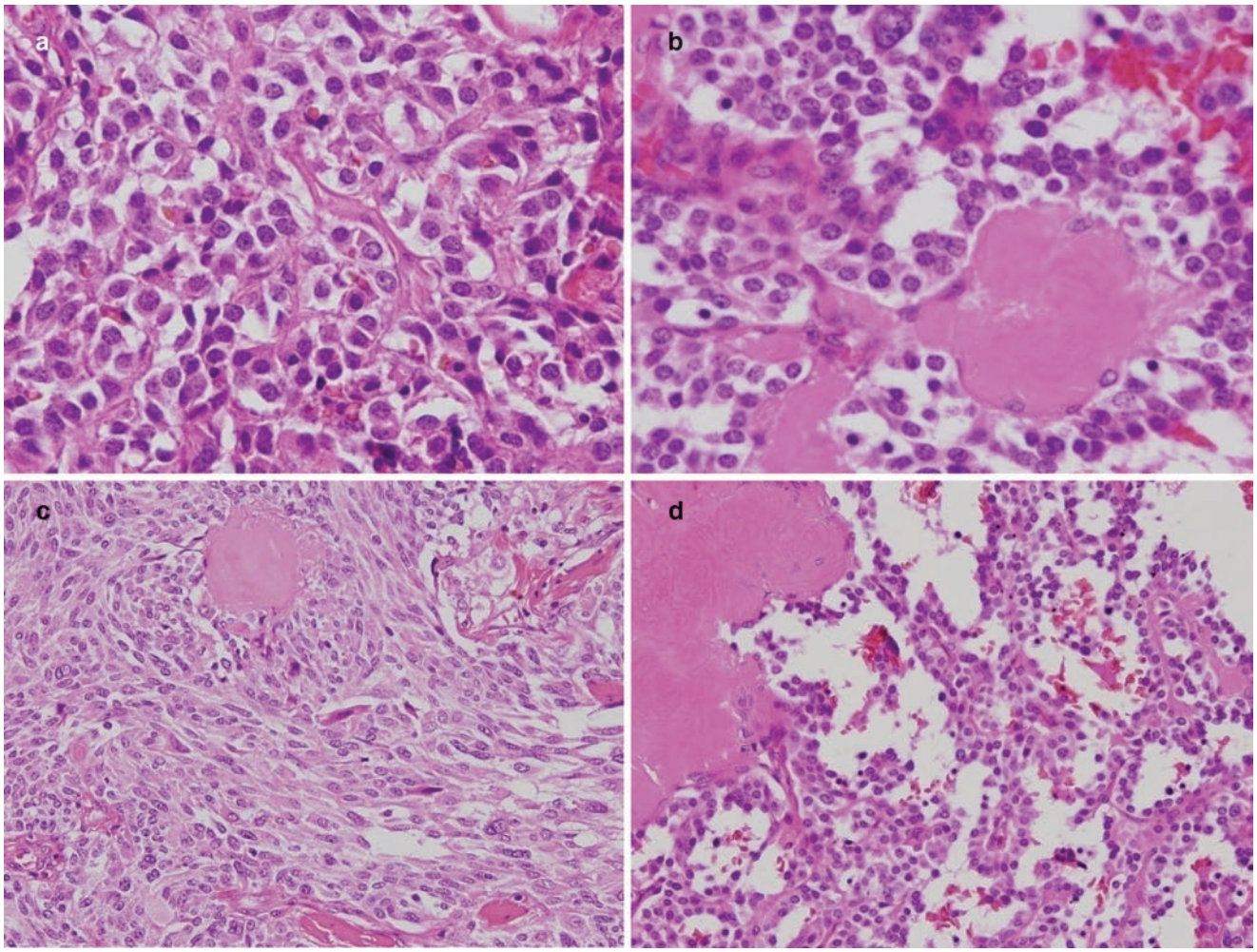

Fig. 24.9 Medullary carcinoma of the patients with MEN 2A patients. **a** Solid growth composed of polygonal cells with vesicular nuclei is shown. **b** Amyloid stroma in solid growth is noted, **c** Spindle cell type of MTC. Note amyloid deposits in the tumor nests. **d** Papillary type MTC containing amyloid deposits in the upper left field

nucleus and slightly eosinophilic granular cytoplasm (Fig. 24.9). They are polygonal and usually have an intermediate amount of cytoplasm with a small-to-intermediate N/C ratio (Figs. 24.9a, b). Amyloid stroma is found in most MTCs (Fig. 24.9b–d) and tumor calcification, such as a psammoma body type in tumor nests, or coarse calcification in the stroma is often observed.

Several markers, such as calcitonin, calcitonin gene-related peptide, adrenocorticotropic hormone, histaminases, chromogranin, neuron-specific enolase, thyroid transcription factor-1, and carcinoembryonic antigen have been reported useful for immunohistochemical diagnosis in MTC (137–144). Ectopic Cushing's syndrome and plurihormone production have been reported in patients with MTC, but only three cases with MEN 2A syndrome in the literature (145–147). Ectopic Cushing's syndrome in patients with MTC is a rare complication (0.6%) of MTC, but the prognosis is poor because of frequency of metastasis at diagnosis (146).

Several histologic variants have been reported in MTCs, such as spindle cell (Fig. 24.9c), ribbon, trabecular, amyloid rich, papillary (pseudo-papillary) (Fig. 24.9d), follicular (tubular), rosette, angiomatous and melanin-producing, etc., and none of these are regarded as indicating poor prognosis (120, 121, 123, 127). Oxyphilic cell change and squamous cell metaplasia in MTCs have been reported as significant features of lower survival (131), but these histological features were rare in our series and could not be confirmed in our experience (unpublished data).

The prevalence of MTC in MEN 2 patients is almost 100%, but the prevalence of MEN syndrome varied from 20 to 40% in all hereditary and sporadic MTCs. It has been suggested that multiple C cell hyperplasia of the thyroid is a hallmark of MEN 2; however, it is of note that proliferation of C cells may be seen in non-MEN patients and sometimes in normal individuals. Guyetant et al. reported C cell clusters in 14 of 42 normal adult autopsy thyroid glands and stated

that C cell density between sexes was different (male > female) and the sex must be taken into account in C cell quantification and assessment of C cell hyperplasia (13). It is our opinion that C cell clusters with small tumors (larger than 1 mm, grossly detectable) (Figs. 24.4 and 24.10) should be regarded as precursor lesions (medullary carcinoma in situ or early stage) of hereditary MTC, and small C cell clusters without clear-cut invasive growth or less than 1 mm should not be diagnosed as neoplastic C cell hyperplasia of MEN 2 syndrome *without* any other indications of MEN 2 syndrome. In such cases, we should wait for either evidences of other tumors of MEN 2 syndrome or the demonstration of *RET* germline mutation to reach a conclusion of neoplastic C cell hyperplasia, early stage of MTC or micro-MTC of MEN 2 syndrome. In Japan, family members with MEN 2 syndrome at risk of germline mutation of *RET* are advised to have thyroid surgery, usually after detectable small thyroid tumors develop, and the total thyroidectomy of young children before age 5 years is extremely rare; therefore, Japanese pathologists have little experience, compared to Western pathologists, of diagnosing early MTC patients with no gross tumors in the thyroid gland, and the description of C cell hyperplasia in this chapter is obtained from C cell lesions observed in MEN 2 patients with gross MTC tumors.

The prognosis of medullary microcarcinoma (less than 1 cm in diameter) in MEN 2 syndrome is much better than that of larger MTCs, and there was no mortality in the32 patients with hereditary microcarcinoma who were followed for 20 years (130).

24.5.4 Prognostic Classification and Proposal of Poorly Differentiated MTC

MTC is regarded as a low-grade malignant tumor originating from C cells of the thyroid and is an example of well-differentiated neuroendocrine carcinoma, such as carcinoid tumor of the lung or neuroendocrine tumor of the pancreas. After surgical treatment, the 10-year survival rate of MTC has been reported as high as 75% (124–133). Several useful prognostic factors have been reported to differentiate the aggressive group from the indolent MTCs. These factors are: (1) the presence of atypical or anaplastic morphologic features (148–151), (2) less-intense calcitonin immunostaining in aggressive cases as compared to diffusely intense staining in common type tumors (131, 136, 152), (3) tumor stage (125–132), (4) tumor with MEN 2B syndrome or sporadic cases (124, 126, 132, 153, 154), and (5) doubling time of tumor markers such as calcitonin (155, 156). From our group, high-risk MTC defined with pure morphological parameters and clinical variables has been reported (136, 156). The histologic characteristics for high risk MTC were composed of vascular invasiveness, high proliferation rate such as high MIB-1 labeling, increased mitoses and tumor necrosis (Fig. 24.11), and cellular dedifferentiation of the increased N/C ratio, less intense immunohistochemical stain for calcitonin and poorly differentiated cytoplasmic organelles (fewer and smaller neurosecretory granules) under electron microscopy. Franc et al. analyzed 109 cases of MTCs, only probands, from the French Calcitonin Tumor Study Group, because

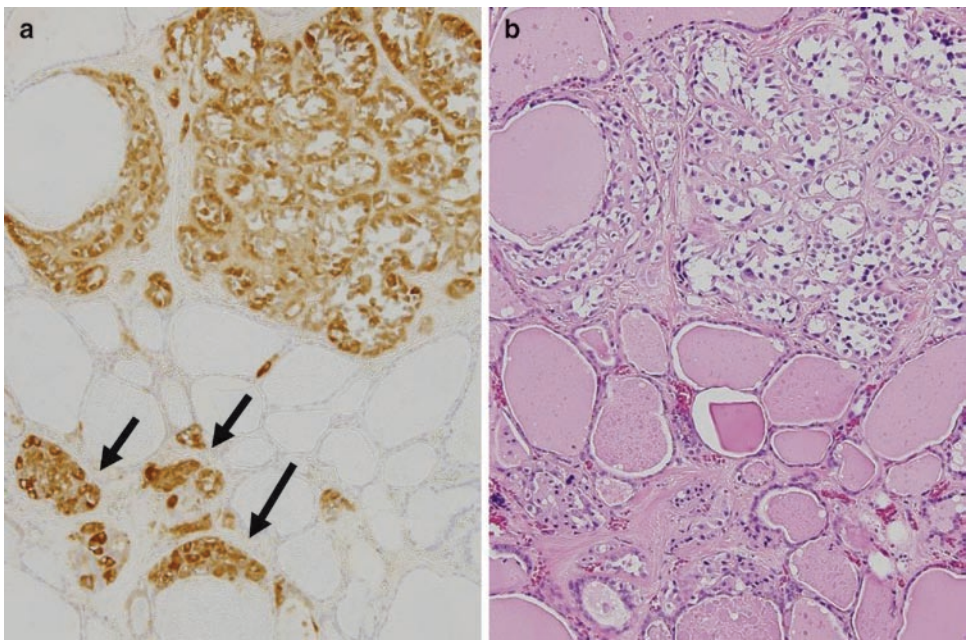

Fig. 24.10 C cell hyperplasia (arrows) and C cell micro-carcinoma (*upper right*) are shown. **a** Immunohistochemical stain for calcitonin and **b** HE stain

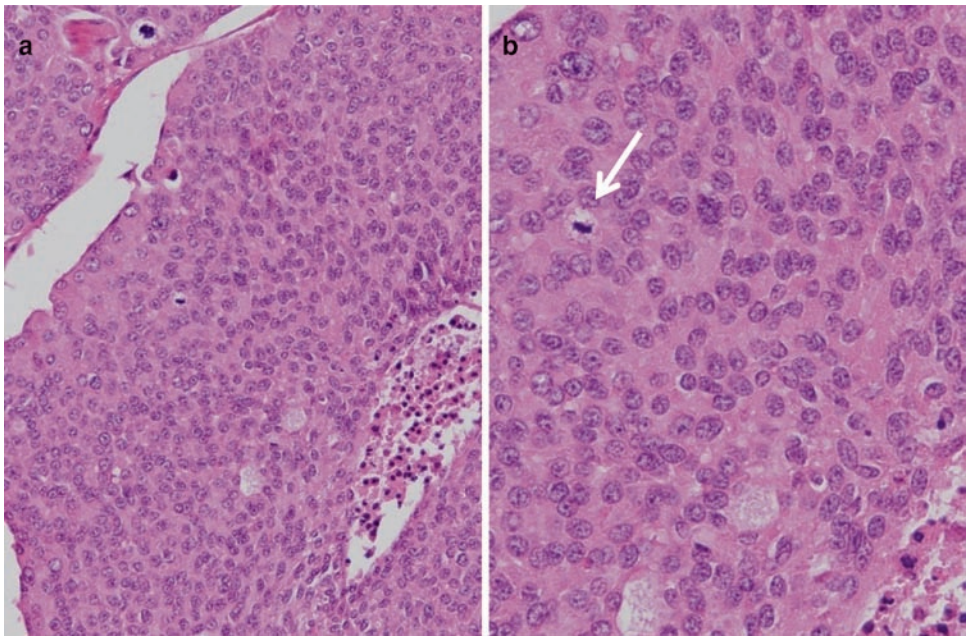

Fig. 24.11 Poorly differentiated MTC shows solid growth with tumor necrosis in **a**. Note increased N/C ratio with slightly basophilic cytoplasm. **b** A mitotic figure (*arrow*) and high nuclear grade are noted in a higher magnification

most previous publications included screen-based early genetic MTCs, which might include serious selection bias in the prognostic results (131). They extracted five parameters as significant, which were confirmed by multivariate analysis, and their results were in good agreement with our conclusions. They were age older than 45 years ($p=0.004$), presence of necrosis in the tumor ($p=0.001$), squamous pattern ($p=0.002$), oxyphilic cells without cells with intermediate cytoplasm ($p=0.025$), and less than 50% of calcitonin immunoreactive cells in the tumor ($p=0.04$).

Age and gender proved to have prognostic impact: the younger age group (<45 years) with MTC had better survival than the older age group and female patients with MTC had better survival than male patients. Several more clinical characteristics of high-risk MTCs have been reported including MTCs with MEN 2B syndrome (124, 126, 132, 153, 154), but this was not reproduced in Franc's series (131).

In conclusion, parameters linked to a rapid tumor growth rate, such as increased mitotic figures, tumor necrosis, high MIB-1 labeling index as well as MTCs with shorter doubling time of plasma calcitonin, and less differentiated C cell features, such as increased N/C ratio (small cell type), less intense calcitonin immunostaining of tumor cells, and poorly differentiated ultrastructural characteristics of tumor cells proved to have more aggressive behavior (136, 155, 156). In our previous proposal, the above features were combined together as poorly differentiated tumor cell features, and this aggressive type of MTC may be designated as poorly differentiated MTC for prognostic purposes, in contrast to common-type MTCs which display well-differentiated C cell features, such as high calcitonin production but longer doubling time, intermediate N/C ratio, rare mitotic figures, no tumor necrosis and rare vascular invasion etc. This prognostic classification of MTCs may reflect well to other classifications of carcinoma with endocrine differentiation in other organs, such as well-differentiated neuroendocrine carcinoma and poorly differentiated neuroendocrine carcinoma of the pancreas (6, 22–24).

24.5.5 Subgroups of MEN 2 Syndrome, MEN 2A, 2B and Familial MTC

MEN2 syndrome consists of three variants, MEN 2A, MEN 2B, and familial medullary thyroid carcinoma (FMTC or MTC-only) (Table 24.5). Because of ganglioneuromatosis and skeletal anomalies, a certain group of MEN 2 patients are designated as MEN 2B, while the majority of MEN 2 patients without ganglioneuromatosis and skeletal abnormality are called MEN 2A (109, 154–164). These two syndromes

Table 24.5 Subgroups of MEN 2 syndrome

1.	MEN2A; medullary thyroid carcinoma, pheochromocytoma parathyroid adenoma (multiple glands)
2.	MEN2B; medullary thyroid carcinoma, pheochromocytoma intestinal and mucosal ganglioneuromatosis characteristic habitus, marfanoid
3.	FMTC; medullary thyroid carcinoma only

share three endocrine neoplasias; multiple MTCs, bilateral pheochromocytoma and multiple parathyroid hyperplasia/adenoma, and have different germline mutations of the same RET protooncogen (4, 157, 158).

Gorlin et al. reported MEN 2B as a new syndrome and reported that parathyroid disease was not generally observed (159). Carney et al. also analyzed parathyroid glands of MEN 2B patients and pointed out adenoma or hyperplasia of the parathyroid is rare or less apparent and recommended more conservative treatment for parathyroid lesions (109, 161). Ganglioneuromatosis in various organs, including the alimentary tract, is one of the manifestations of MEN 2B syndrome in addition to other skeletal abnormalities (109, 159–164).

A certain group of hereditary MTC patients has been known to develop other endocrine abnormalities in low incidence (165, 166). This group of MTC patients usually develops no other manifestations, and is called familial medullary thyroid carcinoma syndrome or the MTC-only family (Table 24.5). In the majority of MEN 2A and FMTC patients, mutations are clustered in six cysteine residues (codons 609, 611, 618 and 620 in exon 10, and codons 630 and 634 in exon 11) in the RET cysteine-rich extracellular domain. These mutations have been detected in about 95 and 85% of MEN 2A and FMTC families, respectively. Moers suggested that MEN 2A families should not be subclassified into MEN 2A and FMTC, but rather according to their specific mutations in the RET protooncogene (166). These patients develop MTC at an older age, the disease tends to be more indolent and in fact develop pheochromocytoma if followed closely for long enough (166).

24.5.6 Overlap Lesions

Medullary thyroid carcinomas have been identified in several cases of MEN 1 patients (23), but this may be not more than a coincidence and detailed gene tests have not been reported in such cases, to the best of our knowledge. Pheochromocytomas, or islet cell tumors, or both have been reported in two or more members of three unrelated families in a manner consistent with an autosomal dominant mode of inheritance (167). At least 14 patients with pituitary adenoma and pheochromocytoma have been reported, and the authors have suggested a new subgroup of MEN syndrome (168).

Mixed medullary and follicular cell carcinoma, which contains two types of tumor cells either C cell differentiation or follicular cell differentiation, was established as a distinct tumor entity in the WHO classification in 2004, but this type of carcinoma is rare and, to our knowledge, it has not been reported in MEN 2 patients (169–171).

24.6 Genetics of MEN 2

24.6.1 Structure of the RET Protooncogene

The *RET* protooncogene was originally identified by transfection of NIH3T3 cells with DNA from a human T-cell lymphoma (172). It is located on chromosome 10q.11.2 and has 21 exons distributed over 60 kb. Analysis of the nucleotide sequence revealed that it encodes a receptor tyrosine kinase with four cadherin-related repeats and a cysteine-rich region in the extracellular domain (Fig. 24.12a) (173, 174). Two major isoforms (short and long isoforms of 1,072 and 1,114 amino acids) generated by alternative splicing in the 3′ region have been identified (175). In 1993 and 1994, germline *RET* mutations were reported in MEN 2A, MEN 2B and FMTC, establishing *RET* as a causative gene in these hereditary syndromes (4, 176–178).

24.6.2 Physiological Functions of RET

RET expression has been detected in human tumors of neural crest origin including neuroblastoma, pheochromocytoma and medullary thyroid carcinoma (179–181). During various developmental stages, it is highly expressed in peripheral enteric, sympathetic and parasympathetic neurons as well as central motor and dopamine neurons. In addition, *RET* expression has been observed in the urinary system, such as the mesonephric duct and ureteric bud during embryogenesis (182, 183). Consistent with this expression pattern, *Ret*-deficient mice showed a lack of enteric neurons in the small and large intestines, a significant decrease in neuron numbers in sympathetic and parasympathetic ganglia, and kidney agenesis or severe dysmorphogenesis (184). These findings demonstrated that *RET* is essential for the development of the enteric nervous system and kidney.

The ligands for RET receptor are members of glial cell line-derived neurotrophic factor (GDNF) family ligands. These include GDNF, neurturin (NRTN), artemin (ARTN) and persephin (PSPN). GDNF family ligands signal through a unique multicomponent receptor complex consisting of glycosyl-phosphatidylinositol (GPI)-anchored coreceptor (GFRα1-4) as a ligand-binding component and RET as a signaling component (Fig. 24.12b) (185–188). GDNF, NRTN, ARTN and PSPN use GFRα1, GFRα2, GFRα3 and GFRα4 as the preferred ligand-binding receptors, respectively (189). Following ligand stimulation, the induced RET dimerization leads to autophosphorylation of intracellular tyrosine residues, which subsequently activates downstream signaling pathways such as RAS/MAPK and PI3K/AKT pathways (190).

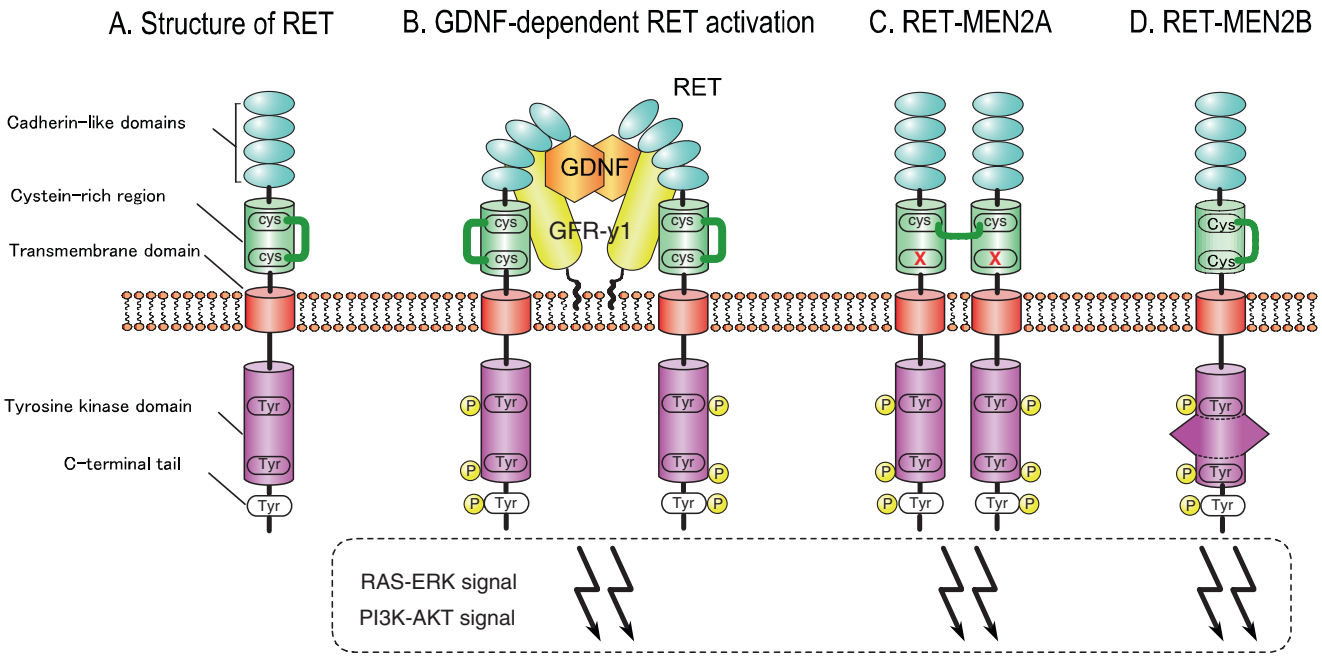

Fig. 24.12 Structure of the RET protein and its activation mechanisms by GDNF or *MEN2* mutations. **a** Schematic representation of the RET tyrosine kinase. The extracellular domain comprises four cadherin-like domains and a cysteine-rich region. **b** Activation mechanism of RET by GDNF. GDNF binds to glycosylphosphatidylinositol-anchored GDNF family receptor α1 (GFRα1) and leads to RET dimerization which activates various intracellular signaling including RAS-ERK and PI3K-AKT pathways. **c** RET activation by *MEN 2A* mutation. Cysteine mutation in the extracellular domain induces ligand-independent RET dimerization by forming intermolecular disulfide bond between mutant proteins. **d** RET activation by *MEN2B* mutation. The *MEN2B* mutation induces a conformational change of the kinase domain, leading to constitutive activation of kinase activity

24.6.3 Germline Mutations of the RET Gene in MEN2

Germline missense mutations have been identified in MEN 2A, MEN 2B and FMTC (4, 176–178). In the majority of MEN 2A and FMTC patients, the mutations are clustered in six cysteine residues (codons 609, 611, 618 and 620 in exon 10, and codons 630 and 634 in exon 11) in the RET cysteine-rich extracellular domain(Fig. 24.13). These mutations have been detected in about 95 and 85% of MEN 2A and FMTC families, respectively. Codon 634 mutations of the cysteine mutations were found in about 85% of MEN 2A families, whereas in FMTC families, about 30% of mutations occurred at codon 634 and more than 60% at codons 609, 618 and 620.

In addition, missense mutations at codons 768, 790, 791, 804 and 891 in the RET tyrosine kinase domain were reported in FMTC or MEN 2A families (Fig. 24.13) (191, 192). In rare cases of MEN 2A of FMTC, a 9- or 12-base pair duplication in exon 11 and a 9-base pair duplication in exon 8 that create an additional cysteine were found (193–195). Moreover, rare missense mutations (codons 321, 533, 600, 603, 606, 649, 666, 777, 778, 781, 852 and 912) have been detected at both extracellular and intracellular domains (192).

Two germline mutations at codon 918 (exon 16) or codon 883 (exon 15) were associated with MEN 2B (Fig. 24.13) (192). Methionine at codon 918 and alanine at codon 883 were replaced with threonine and phenylalanine, respectively. Methionine 918 mutation and alanine 883 mutation accounted for 95% and fewer than 4% of MEN 2B patients, respectively. In addition, double germline mutations at codons 804 and 806 were identified in a Japanese MEN 2B patient (196).

24.6.4 Somatic RET Mutations in Sporadic Tumors

One- to two-thirds of sporadic cases of MTC have been reported to harbor a *RET* mutation. Somatic methionine 918 mutation has been detected in sporadic MTCs. Similarly, in 10–20% of sporadic pheochromocytomas, somatic methionine 918 mutation has also been found. These findings indicated that the *RET* mutations are associated with not only hereditary MEN 2 but also sporadic cases of MTC and pheochromocytoma (197, 198).

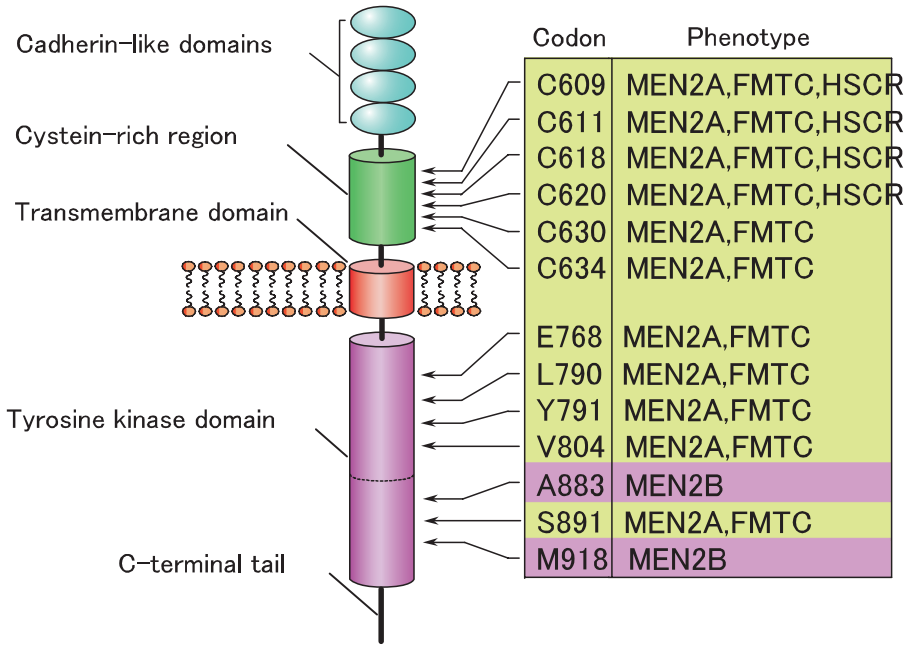

Fig. 24.13 Representation of mutated codons in MEN 2 subtypes

24.6.5 Effect of RET Mutations on RET Protein Function

Biochemical and cell biological analyses revealed that *MEN2A, MEN2B* and *FMTC* mutations induce constitutive activation of RET protein. Thus, *RET* represented the first case in which gain-of-function mutations of a proto-oncogene were implicated in the etiology of an inherited cancer syndrome. Cysteine mutations identified in MEN 2A and FMTC induce a ligand-independent RET homodimer, resulting in constitutive activation of mutant proteins (199, 200). It is thought that when a cysteine in the RET extracellular domain was mutated to a non-cysteine residue, a partner cysteine involved in an intramolecular disulfide bond could become free and form an intermolecular disulfide bond between two mutant RET proteins (Fig. 24.12c).

For *MEN2B* mutations identified in the kinase domain, RET appears to be activated in a monomeric form, probably due to conformational change of catalytic core of the kinase domain (Fig. 24.12d) (200, 201). Several reports suggested that *MEN2B* mutations alter the substrate specificity of RET protein (202), although its activation mechanism is still controversial. RET with the *MEN2B* mutation has high transforming activity compared with RET with *MEN2A* or *FMTC* mutation (203), consistent with the fact that MTCs in MEN 2B patients show aggressive behavior.

Interestingly, it has been reported that MEN 2A/FMTC and Hirschsprung's disease (HSCR) co-segregate in a sizeable fraction of families (204, 205). Screening of the *RET* gene in these families led to the identification of germline missense mutations at either cysteine 609, 611, 618 or 620 (Fig. 24.13). As mentioned above, these cysteine mutations promote the aberrant formation of a disulfide-linked RET homodimers, causing constitutive activation; however, these mutations also caused a strong reduction of RET expression at the plasma membrane and might have altered the folding of RET, partially interfering with RET maturation, intracellular trafficking or stable expression at the plasma membrane. Although the levels of dimerized mutant RET (cysteine 609, 611, 618 or 620 mutant) at the cell surface are low, their activity could be sufficient to trigger the development of MTCs. On the other hand, the decrease of RET cell surface expression in enteric neuroblasts during embryogenesis would transmit a signal below the threshold necessary for complete development of the enteric nervous system in the distal colon (Fig. 24.14). Both gain-of-function (MEN2A/FMTC mutation) and loss-of-function (HSCR mutation) effects could be ascribed to the same mutation in the *RET* gene (206–208).

24.6.6 DNA Test for MEN 2

Genetic screening for the *RET* gene has been shown to be an extremely sensitive marker to confirm the diagnosis of MEN 2. Screening using pentagastrin or the combined

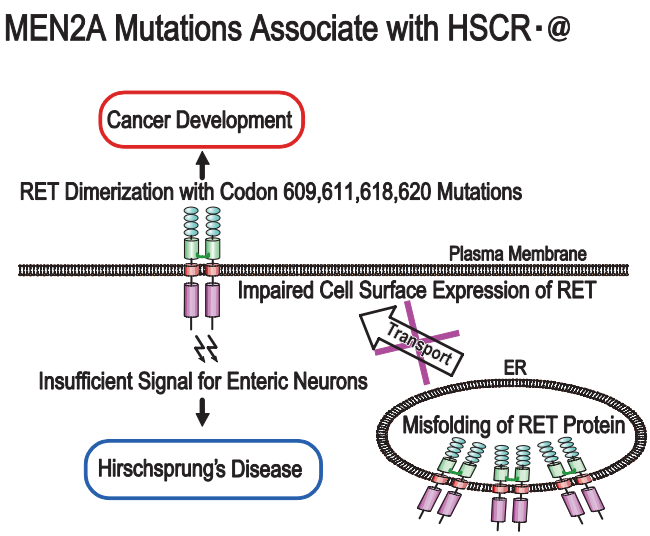

Fig. 24.14 Hypothetical model of development of both MEN 2A and Hirschsprung's disease by cysteine 609, 611, 618 or 620 mutation. These cysteine mutations cause not only ligand-independent RET dimerization but also a reduction of RET expression at the plasma membrane. Reduced RET expression at the plasma membrane of enteric neuroblasts during embryogenesis results in incomplete development of the enteric nervous system in the distal colon

pentagastrin-calcium provocative test sometimes generated a false-positive or a false-negative result. Accurate diagnosis by *RET* testing will release non-mutation carriers from further unnecessary clinical investigations and fear of the disease. DNA test should include exons 10, 11, and 13–16 of the *RET* gene.

Based on the results of DNA tests, effective management, including prophylactic thyroidectomy, will be undertaken before any clinical and biochemical abnormalities. Guidelines for the timing of prophylactic thyroidectomy recommend that prophylactic thyroidectomy should be performed within the first year of life in patients with *MEN2B* mutations and prior to the age of 5 years in patients with MEN 2A or FMTC who have mutations of codons 611, 618, 620, or 634. On the other hand, the timing of surgery for patients with other mutations can be individualized.

In addition, it is clear that cysteine 609, 611, 618 and 620 mutations in *RET* should be screened in HSCR to identify those at risk of developing MEN 2A or FMTC.

24.6.7 Clinical Aspects: MEN 2

MTC originates from calcitonin-producing cells (C-cells) of the thyroid. Although its prevalence is comparatively rare, 1.4% of all thyroid malignancies in Japan and 3–10% of those in Western countries, it is the most common disease in patients with all variants (MEN 2A, 2B and FMTC) of MEN 2 showing complete penetration of these patients (Table 24.2 and 24.5)(209, 210). It is well-known that elevation of serum CEA and calcitonin greatly contributes to the diagnosis and can be a trigger to identify MEN 2 patients by *RET* gene mutation analysis, because it is the first clinical manifestation in most MEN 2 patients. Since MTC shows rather high morbidity, the management of MTC is very important for MEN 2 patients.

24.6.8 Clinical Aspects: Diagnosis of and Treatment Strategies for Clinical MTC

The recent prevalence of ultrasonography for mass screening has facilitated the detection of small and unpalpable thyroid nodules, and development of ultrasonography resolution has enabled qualitative analysis of thyroid nodules. Not only non-hereditary MTC but also hereditary MTC without an apparent family history are most likely found on ultrasonographic examination. Usually, MTC, whether hereditary or not, showed a solid and irregular-shaped nodule with low internal echo, but it is very difficult to discriminate MTC from other thyroid carcinomas, such as papillary carcinoma (Fig. 24.15). Furthermore, some MTCs show ultrasonographic findings that resemble follicular tumor, resulting in misdiagnosis (Fig. 24.16). The next step in diagnosis is fine-needle aspiration biopsy (FNAB). There are specific cytological findings such as a dispersed cell-pattern of polygonal or triangular cells, azurophilic cytoplasmic granules and amyloid (211, 212), although the diagnosis of MTC on cytology is also often difficult. When MTC is suspected on cytological findings, measurement of serum calcitonin is mandatory (213). Furthermore, calcitonin measurement in the wash-out of needles used for FNAB also contributes to diagnosis (214).

The *RET* gene mutation analysis should be performed for all patients diagnosed with MTC. If *RET* gene mutation is detected, adrenal medullary function and imaging studies for adrenal tumor are necessary. If pheochromocytoma is present, it should be treated before surgery for MTC to avoid perioperative complications such as blood pressure fluctuation. Total thyroidectomy should be performed for hereditary MTC, because tumors occur multicentrically. In contrast, total thyroidectomy is not mandatory for non-hereditary MTC, although it is also routinely performed in Western countries. This is because recurrence in the remnant thyroid is a rare event in non-hereditary MTC (215). Extensive lymph node dissection is necessary for MTC surgery, regardless of whether it is hereditary or not. Unlike papillary and follicular carcinomas, radioiodine ablation therapy and thyroid-stimulating hormone (TSH) suppression therapy are not effective for MTC patients; therefore, surgical curability must be more strictly pursued than for other thyroid malignancies. In many institutes, dissection of only of the central compartment is still

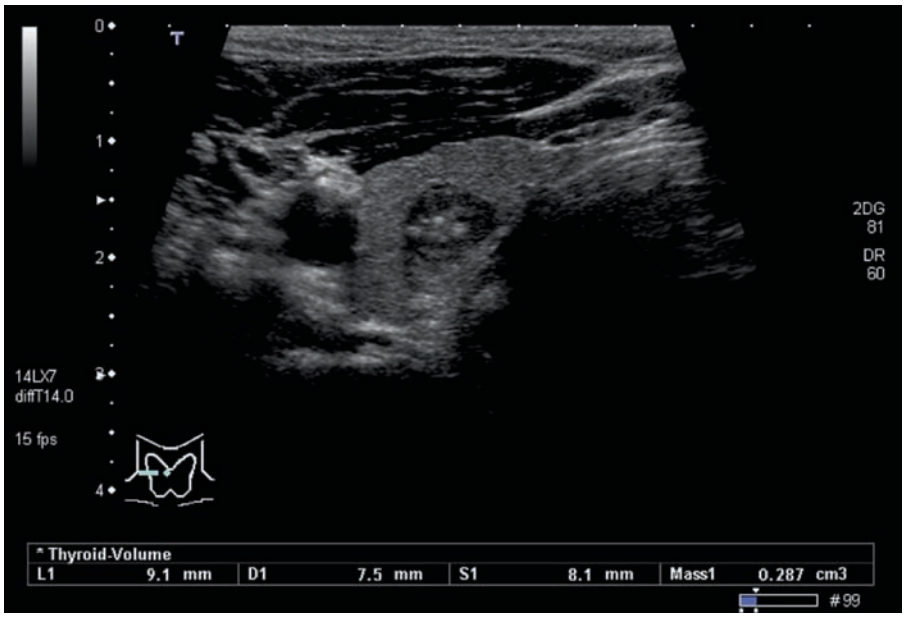

Fig. 24.15 Ultrasonotraphic profiles of medullary thyroid carcinoma. This nodule shows a similar ultrasonographic profile of papillary carcinoma

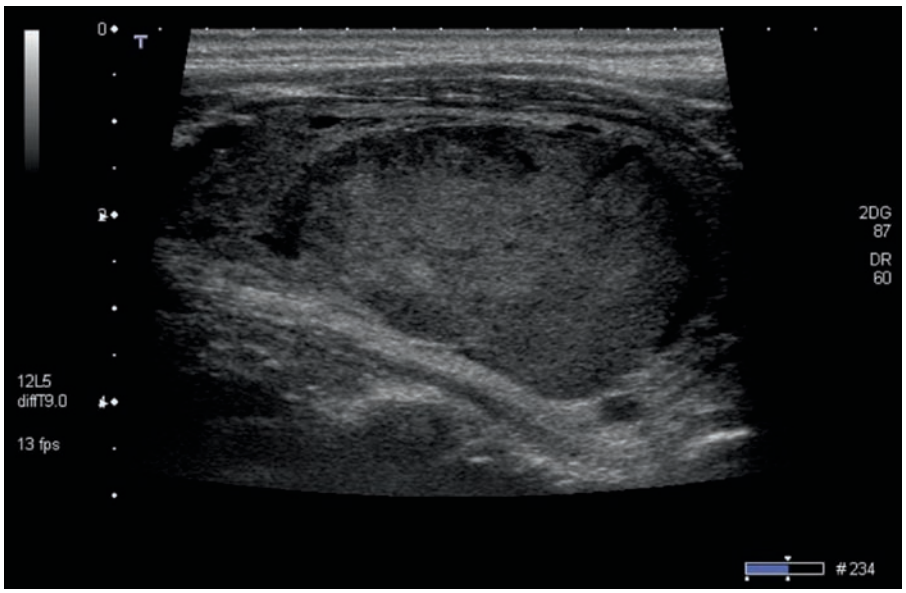

Fig. 24.16 Ultrasonotraphic profiles of medullary thyroid carcinoma. This nodule is likely to be misdiagnosed as follicular tumor

performed but, just like papillary carcinoma, the prevalence of metastasis to the lateral and central compartments is similar in MTC; furthermore, as high as 40% of MTC of 1.0 cm or smaller showed lateral node metastasis. This prevalence increased with tumor size and 86% of MTC larger than 4 cm were lateral node-positive (215). Thus, modified radical neck dissection (MND) should be performed at least on the ipsilateral side of the primary lesion, even if it is prophylactic.

Whether the surgery is curative is determined by calcium stimulation test performed about 1 week after surgery (216, 217). In the past, calcium and tetragastrin were used for the test, but the latter is now unavailable. When basal calcitonin is within the normal range and the peak calcitonin level is less than threefold greater than basal calcitonin, the patient is judged as being biochemically cured (203, 217). Interestingly, the calcitonin level does not always decrease to below measurement sensitivity even after total thyroidectomy. This is possibly because calcitonin is secreted from other organs (218). The incidence of biochemical cure in a recent study was 68%, but other reports presented lower percentages (215);

thereafter, serum CEA and calcitonin levels should be measured two to four times per year to investigate whether carcinoma has recurred. In patients with elevated serum CEA and calcitonin levels, recurrence should be assessed on imaging studies but identification of metastatic lesions is often difficult. Second surgery is considered if recurrence is seen in local organs such as regional lymph nodes, including mediastinal nodes. External radiation therapy may be effective for bone metastasis and local recurrence that cannot be surgically dissected. Furthermore, calcitonin and CEA doubling time are important predictors of CSS in patients (155, 219, 220)

Previous studies from western countries demonstrated that the prognosis of MTC patients is somewhat worse than papillary carcinoma: 78–97% at 5-year follow-up and 61–88% at 10-year follow-up (221–232); however, a report from Japan presented better outcomes: 10-year and 20-year cause specific survival of 97% and 92%, respectively (215). As shown in Figs. 24.17 and 24.18, the prognosis of patients with hereditary MTC, except for MEN2B, was excellent. This may be because the biological character of MTC in Japan is milder than in western countries or because of the performance of routine MND even for cases without clinically apparent node metastasis. Various prognostic factors of MTC have been identified in previous studies, such as age, gender, lymph node metastasis, and extrathyroid extension (221–232). It is important to predict patient prognosis by

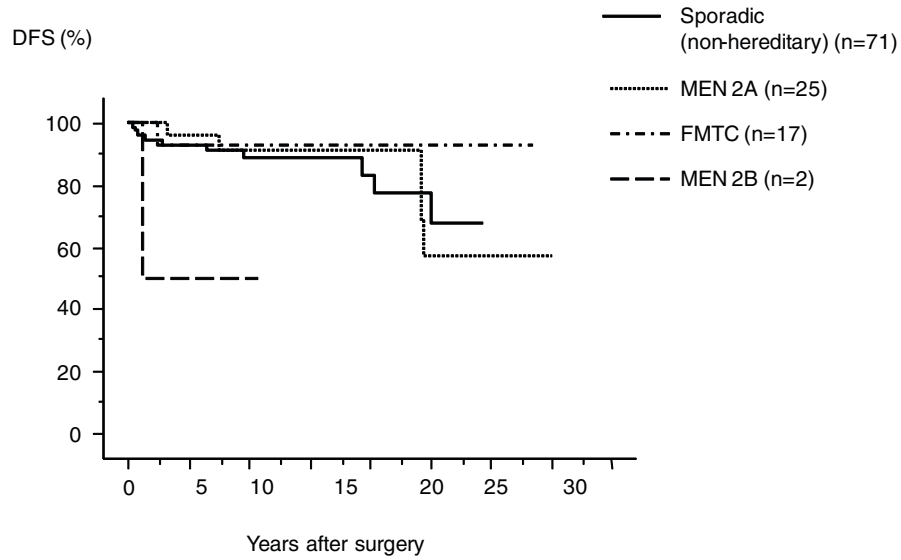

Fig. 24.17 DFS of patient with sporadic nonhereditary MTC, MEN 2A, MEN 2B, and FMTC

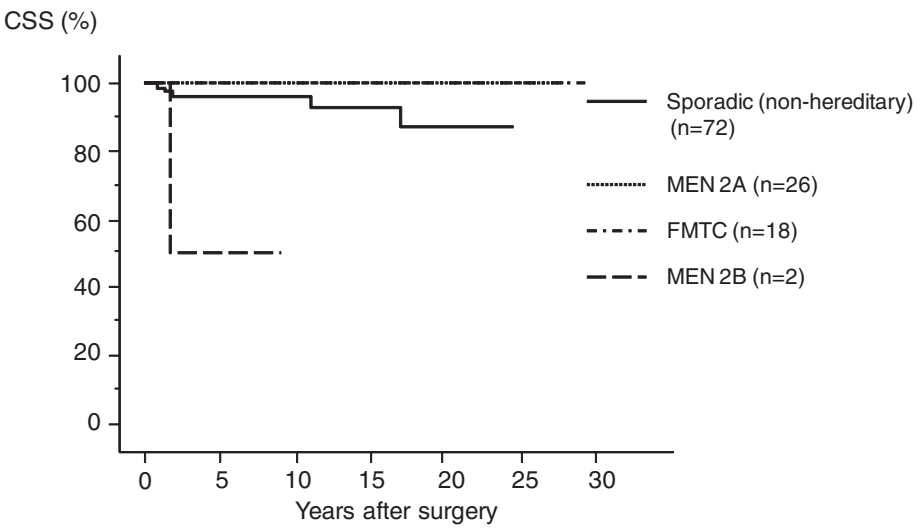

Fig. 24.18 CSS of patients with sporadic nonhereditary MTC, MEN 2A, MEN 2B, and FMTC

whether a patient is biochemically cured. As shown in Figs. 24.19 and 24.20, a patient who was biochemically cured was significantly less likely to develop recurrence or die from carcinoma than a patient without biochemical cure. As shown in Fig. 24.20, only one patient in our series died of carcinoma after being biochemically cured. Furthermore, biochemical cure was significantly related to other prognostic factors such as lymph node metastasis, extrathyroid extension and tumor size (Table 24.6). Lymph node metastasis is another important prognostic factor of MTC patients. In our experience, none of the patients negative for lymph node metastasis showed carcinoma recurrence. Furthermore, none of the patients with no clinically apparent node metastasis that was palpable or detected on preoperative imaging studies died of carcinoma (215). It is notable that, as shown in Table 24.6, 45% of patients who was positive for node metastasis on pathological examination and 27% of those positive for clinically apparent node metastasis on preoperative imaging studies were biochemically cured, indicating that extensive lymph node dissection is important to improve the prognosis of MTC patients. In MTC, extrathyroid extension or extranodal tumor extension is rarer than in papillary

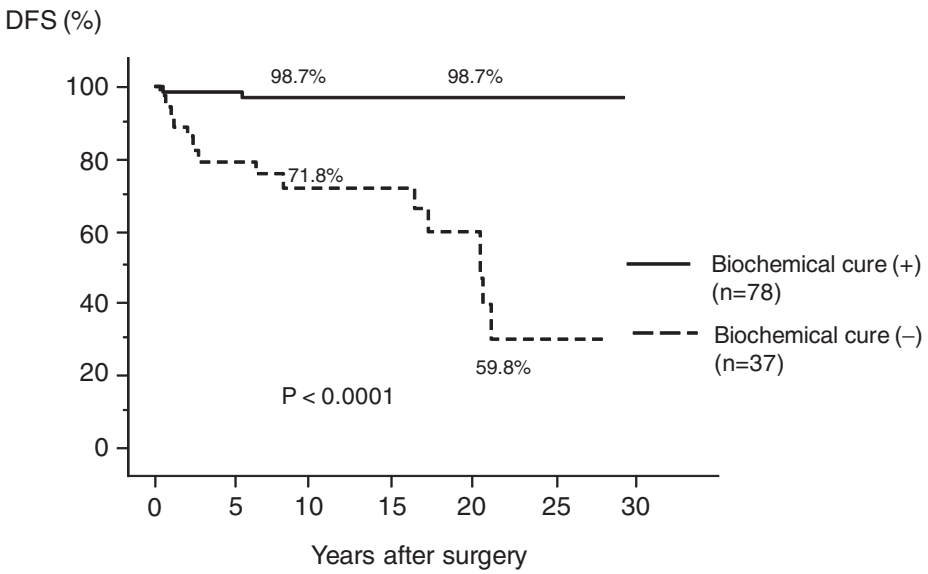

Fig. 24.19 Comparison of DFS between patients with and without biochemical cure

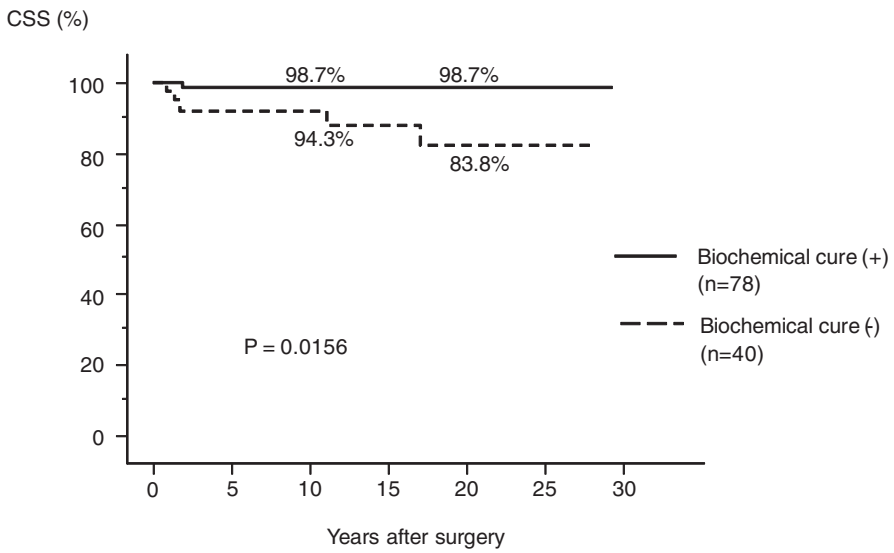

Fig. 24.20 Comparison of CSS between patients with and without biochemical cure

Table 24.6 Relationship between biochemical cure and clinicopathological features

	Biochemical cure (%)			
	Yes	No	Total	*p* values
Lymph node metastasis				
Yes	30 (44.8)	37 (55.2)	67	
No	48 (100)	0	48	<0.0001
Extrathyroid extension				
Yes	4 (30.8)	9 (69.2)	13	
No	74 (72.5)	28 (27.5)	102	0.0042
Pathological extranodal tumor extension				
Yes	2 (20.0)	8 (80.0)	10	
No	76 (72.9)	29 (27.7)	105	0.0017
Tumor size				
>4 cm	5 (38.5)	8 (62.5)	13	
≤4 cm	73 (73.0)	27 (27.0)	100 (unknown)	0.0217

carcinoma, but if present, the prognosis is fearful and 20-year CSS would be around 60% (215).

24.6.9 Prophylactic Surgery for Children with RET Gene Mutations

It is well-known that the biological aggressiveness of MTC varies according to the mutated codon of the *RET* oncogene. Prophylactic total thyroidectomy in childhood is recommended for patients with *RET* gene mutations, but the appropriate time of surgery varies for mutated codons. Children with a *RET* gene mutation are stratified into three levels according to the risk level and aggressiveness of MTC. Children with ret codons 883, 918 or 922 mutations are classified as level 3, because these mutations show MEN2B as a clinical syndrome. These patients should undergo total thyroidectomy, preferably in the first month of life or as soon as the mutations are identified. Not only MTC lesions in the thyroid but also lymph node metastasis are detected within the first year of life in these patients (164, 233–236); therefore, at least total thyroidectomy with central node dissection is recommended.

Children with codon 634 mutation can have microscopic MTC at the age of 2 years and lymph node metastasis at 5 years (237, 238). According to Nunziata et al., prophylactic total thyroidectomy should be performed by the age of 6 years, and the guideline recommends surgery before the age of 5 years (66). There is little consensus whether central node dissection is needed for these patients. According to the EUROMEN consortium report, no patients with mutation of codon 634 had node metastasis below age 14-years (239); however, central node dissection can be performed via the same wound as total thyroidectomy and reoperation of this lesion for recurrence has a high risk of severe complications, such as recurrent laryngeal nerve injury and persistent hypoparathyroidism; therefore, central node dissection should be performed at the same time as total thyroidectomy. Children with mutations of codons 611, 618 and 620 are also classified into the same group as those with codon 634 mutation, level 2.

Level 1 includes children with *RET* gene mutation at codons 609, 768, 790, 791, 804, and 891. Generally, MTC in level 1 children is more indolent and slow-growing. Death from MTC in patients with mutations of codons 790 and 791 has not been reported. There is no consensus on the thyroid management of patients with level 1 mutations. There are various recommendations that thyroidectomy should be performed by the age of 5 years, or 10 years, or when the calcium-pentagastrin stimulating test shows abnormal calcitonin levels (66).

24.6.10 Targeted Therapy for Metastatic MTC

Carlomagno and associates showed in an in vitro model that the tyrosine kinase inhibitor ZD6474 blocks oncogenic RET kinases (240). In a phase II clinical trial of, 20 cases of locally advanced or metastatic MTCs, decrease of plasma CEA and calcitonin were noted in 60 and 70% of patients, respectively, and objective remissions in up to 30% (241).

24.6.11 Clinical Aspects: Pheochromocytoma in MEN2 Patients

It is known that pheochromocytoma develops in 50% of MEN 2 patients at maximum and in tumors (110). It can coexist with von Recklinghausen's disease and von Hippel-Lindau disease with a prevalence of 5% and 10–20%, respectively (242, 243). Phochromocytoma in MEN 2 patients can be unilateral or bilateral, and its entity is hyperplasia of the adrenal medulla (110, 244, 245). The most important problem is hypertensive crisis of unconscious pheochromocytoma that causes sudden death of patients, although morbidity from this disease is markedly decreased by improving the management. Pheochromocytoma can be screened by measuring plasma metanephrines, urinary catecholamines or metanephrines (246). If any show high levels, imaging studies such as CT scan and MRI are necessary to identify adrenal tumors. In addition, before surgery for MTC in patients with *RET* gene mutations, these laboratory studies and, if possible, imaging studies are routinely recommended.

Before surgery for pheochromocytoma, pharmacotherapy using alpha- and beta- adrenergic antagonists and/or alpha-methyltyrosine is necessary for safety. Laparoscopic adrenalectomy is a useful strategy for a unilateral tumor, whereas open surgery can be selected for bilateral tumors. Especially for patients who have undergone bilateral adrenalectomy, special care against adrenal insufficiency is necessary. Parental corticoid therapy is necessary in an emergency.

Pheochromocytoma was found in all family members except in codons 609, 768, val804met and 891 (66). Screening for pheochromocytoma should start by the age of 5–10 years, especially in patients with mutations in codons of levels 3 and 2, although the family pattern of pheochromocytoma should be considered. Screening should be performed annually. For patients with mutations in low-risk codons, screening can be initiated at an older age.

24.6.12 Clinical Aspects: Hyperparathyroidism

Clinically apparent hyperparathyroidism is rare in MEN 2 patients, but when performing surgery, all parathyroid glands should be identified. Indications for surgery are the same as for primary hyperparathyroidism of multiple glands (247–251). For patients with a mutation at codon 634, screening for hyperparathyroidism is recommended annually. For other patients, investigation every few years is adequate.

24.6.13 Clinical Aspects: Ganglioneuromatosis in MEN 2B

Ganglioneuromatosis in various organs is regarded as a hallmark of MEN 2B syndrome (159–166) and is also found in the lips, oral cavity, salivary glands, stomach, intestine, colon, rectum, pancreas, gall bladder, upper respiratory tract, and urinary bladder of MEN 2B patients (109, 160). Other than the alimentary tract, it is also found in the cornea as a beaded thick nerve and in the subcutis, and probably in most organ systems. Early identification of MEN 2B patients is easy from abnormal phenotypes, such as bumpy lips and marphanoid habitus (159–161). This is clinically important because MEN 2B patients have specific symptoms related to ganglioneuromatosis, such as constipation, diarrhea, difficulty with feeding, vomiting, abdominal pain, abdominal distention and roentgenographic evidence of megacolon.

This abnormality in the alimentary tract may cause acute abdomen due to megacolon and functional ileus (161, 166). It is important for clinicians to recognize this alimentary tract ganglioneuromatosis in MEN 2B patients, not only because it is present before endocrine neoplasms are detected, but also because patients may present with intestinal obstruction as early as several days or weeks after birth (166).

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Chapter 25
Polyglandular Autoimmune Diseases

Ricardo V. Lloyd

25.1 Introduction

Polyglandular autoimmune syndrome which is now more commonly referred to as autoimmune polyendocrine syndrome (APS) includes a complex mixture of endocrine and non-endocrine disorders. APS is generally divided into type I and type II disorders (Table 25.1) [1, 2].

25.2 APS Type I

APS type I is also termed autoimmune polyendocrinopathy candidiasis-ectodermal dystrophy (APECED) but is more commonly referred to as APS I. Patients with APS 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]. As the disorder may appear at intervals of 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 APS I [1].

More variable endocrine features also include Hashimoto's thyroiditis, oophoritis, type I diabetes mellitus and lymphocytic hypophysitis [5].

Patients with APS I usually have antibodies against the parathyroid and adrenal glands [6]. Antibodies against 17-hydroxylase (CYP17) and side chain cleavage enzyme have been reported in Addison's disease associated with APS I [7–9]. This is in contrast to APS II, in which autoantibodies against 17-hydroxylene (CYPI7) and side chain cleavage enzyme (CYP11A1) are commonly found [7, 8].

Most patients have antibodies to glutamic acid decarboxylase (GAD65). This antibody may be detected up to 8 years before the development of diabetes mellitus.

25.3 Genetics

APS I is inherited as an autosomal recessive disorder. There is a 25% recurrence risk for siblings of affected patients [10]. The disease is highly prevalent in Finland and parts of Italy [11]. The molecular genetics of APS I has been elucidated [12–15].

The autoimmune regulator (AIRE) gene is located on chromosome 21q 22.3. More than 60 different mutations in the AIRE gene have been identified [13] 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 [14]. 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 APS I [14]. A recent study examined mutations of AIRE l 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 [15]. 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 AIRE l mutations were so rare in the general population that they could not contribute to the susceptibility of the more common isolated autoimmune disorders [15].

Animal models have been used to study AIRE [5, 17]. A mouse model the G228wknockin mouse has been used in a dominant-negative manner to cause a unique autoimmunity

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Table 25.1 Autoimmune polyendocrine syndromes – endocrine tissues that are frequently involved (Compiled from [1, 2]).

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

[a]Other conditions associated with APS I include chronic active hepatitis, malabsorption, oral squamous cell carcinoma, alopecia, vitiligo, oophoritis, lymphocytic hypophysitis, pernicious anemia, pure red cell hypoplasia, myopathy, keratopathy and asplenism

[b]Other conditions associated with APS II include: celiac disease, alopecia, vitiligo, dermatitis herpetiform, pernicious anemia, idiopathic thrombocytopenia, purpura, myasthenia gravis, Parkinson's disease, IgA deficiency and Goodpasture syndrome

syndrome which led to inhibition of a large number of AIRE-regulated thymic antigens [16]. In this model, there was an autosomal dominant mechanism.

Recent studies in patients with APS 1 have shown that there is a tissue-specific autoantigen involved in hypothyroidism [18]. This protein is known as NACTHT leucine-rich repeat protein 5 of NALP5. NALP5-specific antibodies were found in the serum of about half of patients (59%) with APS 1 [17]. Autoantibodies against NALP5 appear to be highly specific and it has been suggested that these autoantibodies may be diagnostic for this prominent component of APS1 [18].

In a recent study of autoimmune Addison's disease, it was shown that there was a large deletion of the AIRE gene covering at least exon 2 to exon 8. These mutations were associated with APS1, and there was no polymorphism associated autoimmune Addison's disease with APS II [19].

25.4 APS Type II

APS II is more common than APS 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 APSI. There is a female preponderance with a peak incidence between ages 20 and 60. It has also been designated as Schmidt's syndrome, polyglandular failure syndrome, and organ-specific autoimmune disease. APS II is often defined by the development of two or more of the following conditions: Addison's disease, Graves' disease, autoimmune thyroiditis, type I diabetes mellitus, myasthenia gravis, celiac disease, or primary hypogonadism. In one study of 224 patients with Addison's disease and APS 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 APS II, unlike in APS I, although it may be present in a small number of older patients [20]. Patients do not develop mucocutaneous candidiasis.

25.5 Genetics

There is no clear pattern of inheritance for APS II, although familial clustering has been noted. Multiple genetic loci, especially HLA, probably determine susceptibility [1]. The HLA haplotypes involved include HLA-AI, HLAB8, HLA-DR3, and HLA-DR4. Interestingly, some HLA alleles are associated with protein from diseases such as the DQ alleles DQAl, DQB2 which protect against diabetes mellitus, but ironically increase susceptibility to multiple sclerosis [21].

Some components of APS II are not associated with HLADR3 [22]. These include pernicious anemia, vitiligo, and multinodular thyroiditis.

The gene responsible for APS II has not been characterized to date but will probably be linked to the HLA loci. Recent studies of the pedigree data of patients with APS II have suggested an autosomal-dominant or a complex mode of inheritance [23]. The phenotypic diversity of APS II may also be influenced by environmental factors such as viral infection, nutritional factors or hormones [23]. The cytotoxic T lymphocyte antigen 4 (CTLA-4) gene has also been implicated in the genetic susceptibility to a number of autoimmune diseases including autoimmune thyroid disease in APS II as well as autoimmune Addison's disease, celiac disease and type I diabetes mellitus.

25.6 Treatment

The treatment of patients with APS 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].

APS II therapy includes treatment of the individual disorders such as diabetes mellitus, Addison's disease, Graves' disease, and hypogonadism [1].

25.7 Miscellaneous APSs

25.7.1 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 3,000 kb. The microdeletions mediated by homologous recombination between low-copy repeated sequences are detected in 1 in 2,000–4,000 live births [24, 25].

25.7.2 Trisomy-21

Trisomy-21 or Down's syndrome is associated with thyroiditis and diabetes mellitus in addition to the other classical findings. These patients also have T-cell abnormalities [26].

25.7.3 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 [27–29].

Patients usually have sensorimotor polyneuropathy, lymphadenopathy, hepatosplenomegaly with plasma cell dyscrasia, and sclerotic bone lesions. The diabetes usually responds to insulin therapy.

25.7.4 Congenital Rubella

Patients with congenital rubella may develop diabetes mellitus, thyroiditis, and hypothyroidism [30, 31]. 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 [32, 33]. Some patients may also develop other autoimmune diseases such as Sjogren's syndrome and systemic lupus erythematosus.

25.8 Summary

The majority of APSs belong to the PGA II group. Although the gene for APS I have been cloned and characterized, this has not yet been accomplished for APS II, but various HLA loci have been implicated in susceptibility to APS II.

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Chapter 26
Biochemical Testing for Neuroendocrine Tumors

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26.1 Introduction

Neuroendocrine tumors (NETs) are rare, slow-growing neoplasms characterized by being able to store and secrete different peptides and neuroamines [1]. Some of these substances cause a specific clinical syndrome [2]. It is important to be able to recognize from the clinical presentation, the most useful markers to reduce time and costs, and that way, facilitate the diagnosis and make wise use of resources. Unfortunately, there is no "ideal neuroendocrine tumor marker" [3], but according to the presentation, the sensitivity and specificity of each marker vary and it is generally possible to choose those of greatest value for each patient. Also, it is important to recognize the contribution of each marker to diagnosis, follow up of treatment response or prognosis.

The biochemical markers are those hormones or amines secreted by the enterochromafin cells from which these tumors are derived. Some of these are nonspecific to any tumor, but in contrast are produced and secreted by most NETs, other biochemical markers are more specific to the type of tumor.

The number of neuroendocrine tumors (NETs) has risen to 40–50 cases per million; probably due to better diagnosis than a change in the real incidence of the disease; but we still need more accurate and precise biochemical methods for trying to diagnose the presence of a NET as accounting for a symptom complex (Fig. 26.1).

The natural history of this disease is invariably attended by a long history of vague abdominal symptoms, a series of visits to a primary care practitioner, and referral to a gastroenterologist, often with a misdiagnosis of irritable bowel syndrome (IBS). These symptoms persist with a median latency to correct diagnosis of 9.2 years by which time the tumor has metastasized, causing symptoms like flushing and diarrhea and progressing on its slow but relentless course until the patient dies. Clearly, a greater index of suspicion and a carcinoid tumor profile screen is warranted for all patients presenting with "traditional IBS symptoms." The diagnosis of metastasis to the liver is generally more obvious but often takes place only after a delay of many years. Even then, an incorrect diagnosis is *not* uncommon. Unless biopsy material is examined for the secretory peptides chromogranin, synaptophysin, or neuron-specific enolase (NSE), tumors may be labeled erroneously as adenocarcinoma, with a negative impact on physician's attitudes regarding management and underestimation of prospects for survival [4].

26.2 Specific Biochemical Markers for Each Tumor Type

Each tumor, depending on the site of origin will be more prone to produce one or another hormone or peptide (Tables 26.1 and 26.2). In rare cases, when the tumor is localized before the symptoms occur, then these biochemical markers will be useful to confirm the diagnosis, follow the progression or treatment response and may even have prognostic value.

26.2.1 Foregut Carcinoid Tumors

These tumors occur in the thymus, bronchus, stomach, first portion of the duodenum, pancreas and ovaries. These tumors produce less serotonin when compared to carcinoid tumors in the midgut and secrete the serotonin precursor 5-Hydroxytryptophan (5-HTP). This is due to a deficiency in dopa-decarboxylase, an enzyme that catalyzes the conversion of 5-HTP to serotonin (Fig. 26.2). However, after secretion, a small amount of 5-HTP is converted to 5-hydroxyindoleacetic acid (5-HIAA) and serotonin, so modest elevation of these metabolites can be found with foregut tumors (Fig. 26.3). When all these metabolites are

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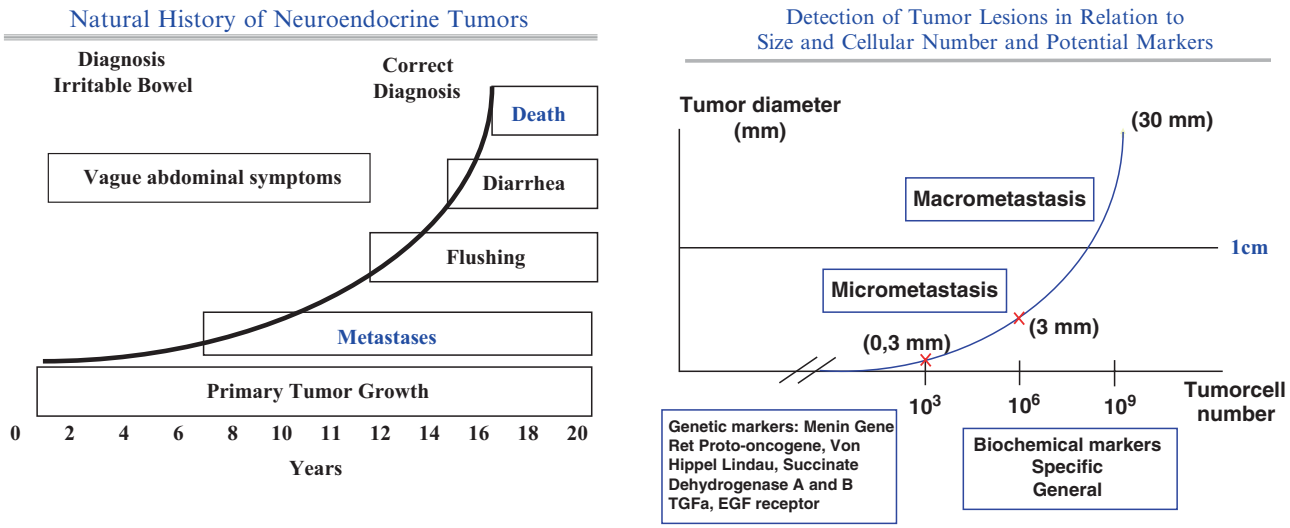

Fig. 26.1 The natural history of neuroendocrine tumors [5]. Vague symptoms such as abdominal pain precede the diagnosis by a median of 9.2 years and flushing and diarrhea the major manifestations of carcinoid NETs occur after the tumor has metastasized. . On the right the figure shows the relationship between tumor size and when the biochemical markers are positive when measured in blood, usually, after the tumor reaches a diameter of approximately 3mm and contains about one million cells

Table 26.1 Specific biochemical markers for each tumor type [6]

Site	Tumor type	Marker	Specificity
All		CgA and B	High
		PP, NSE, Neurokinin, Neurotensin	Intermediate
		HCG α and ß	Low
Thymus	Foregut Carcinoid	ACTH	Intermediate
Bronchus	Foregut Carcinoid, Small Cell Lung Carcinoma.	ACTH, ADH, Serotonin, 5-HIAA, Histamine, GRP, GHRH, VIP, PTHrp	Intermediate Low
Stomach	Foregut Carcinoid, Gastrinoma, Ghrelinoma.	Histamine, Gastrin	Intermediate
		Ghrelin	Low
Pancreas	Gastrinoma, Insulinoma, Glucagonoma, Somatostatinoma, PPoma, VIPoma.	Gastrin, Insulin, Proinsulin, Glucagon, Somatostatin	High Low
		C-peptide, Neurotensin, VIP, PTHrp, Calcitonin	
Duodenum	Gastrinoma, Somatostatinoma.	Somatostatin, Gastrin	High
Ileum	Midgut Carcinoid	Serotonin, 5-HIAA	High
		Neurokinin A, Neuropeptide K, Substance P	Intermediate
Colon and Rectum	Hindgut Carcinoid	Peptide YY, Somatostatin	Intermediate
Bone	Metastasis	Bone Alkaline Phosphatase, N-Telopeptide	High (blastic lesions), Modest (lytic lesions)
		Vitamin D 25, 1:25 OHD	Universal Vit D deficiency
		PTH, PTHrp	Intermediate
Cardiac Involvement	Carcinoid	BNP	Intermediate

Table 26.1 Shows the specific biochemical markers used for each tumor and their specificity. *CgA and B* Chromogranin A and B, *PP* pancreatic polypeptide, *NSE* neuron-specific Enolase, *HCG* human chorionic gonadotropin, *ACTH* adrenocorticotropic hormone, *ADH* anti diuretic hormone, *5-HIAA* 5 hydroxyindoleacetic acid, *GRP* gastrin releasing peptide, *GHRH* growth hormone releasing hormone, *VIP* vasointestinal peptide, *PTHrp* parathyroid hormone related peptide, *BNP* brain natriuretic peptide

measured together, the sensitivity increases to 84%. Other products of foregut carcinoids are histamine, substance P, neuropeptide K, pancreatic polypeptide (PP) and chromogranin A (CgA) [4]. A further point of interest is that a gender variation is present when a carcinoid tumor coexists with MEN-I syndrome; more than two-thirds of the time the tumor is in the thymus in males, whereas in females, more than 75% of the time it is in the lungs [4].

26.2.2 *Midgut Carcinoid*

These tumors occur in the second portion of the duodenum, jejunum, ileum and right colon; they are argentaffin-positive; as opposed to foregut tumors, they produce huge amounts of serotonin but the serotonin precursor 5-HTP is rarely produced. These tumors also secrete other vasoactive compounds like kinins, prostaglandins and substance P [4].

Table 26.2 The clinical presentations, syndromes, tumor types, sites and hormones [4]

Clinical presentation	Syndrome	Tumor type	Sites	Hormones
Flushing	Carcinoid	Carcinoid	Mid/ foregut Adrenal medulla Gastric	Serotonin, NKA, TCT, PP GCRP, VIP Substance P
Diarrhea	Carcinoid, WDHHA, ZE, PP, MCT	Carcinoid, VIPoma, Gastrinoma, PPoma, Medullary carcinoma thyroid	As above, pancreas, mast cells, thyroid	As above, VIP, gastrin, PP, calcitonin
Diarrhea/steatorrhea	Somatostatin Bleeding GI tract	Somatostatinoma, neurofibromatosis	Pancreas Duodenum	Somatostatin
Wheezing	Carcinoid	Carcinoid	Gut/pancreas/lung	SP, CGRP, serotonin
Ulcer/dyspepsia	Zollinger Ellison,	Gastrinoma	Pancreas/duodenum	Gastrin
Hypoglycemia	Whipple's triad	Insulinoma, sarcoma, hepatoma	Pancreas, retroperitoneal liver	Insulin, IGF1, IGF11.
Dermatitis	Sweet Syndrome	Glucagonoma	Pancreas	Glucagon
	Pellagra	Carcinoid	Midgut	Serotonin
Dementia	Sweet syndrome	Glucagonoma	Pancreas	Glucagon
Diabetes	Glucagonoma	Glucagonoma	Pancreas	Glucagon
	Somatostatin	Somatostatinoma	Pancreas	Somatostatin
DVT, Steatorrhea, Cholelithiasis	Somatostatin	Somatostatinoma	Pancreas	Somatostatin
Neurofibromatosis			Duodenum	
Silent, liver mets	Silent	PPOMA	Pancreas	PP
Acromegaly	Acromegaly, Gigantism	NET	Pancreas	GHRH
Cushings	Cushings	NET	Pancreas	CRH, ACTH
Pigmentation	Pigmentation	NET	Pancreas	MSH
Anorexia, nausea, vomiting, abdominal pain	Hypercalcemia	NET	Pancreas	PthRP

The Table 26.2 summarizes our approach based upon the clinical presentation, the tumor type, their sites of origin and the possible means of diagnosis and the biochemical markers that should be measured. *CGRP* Calcitonin gene-related peptide

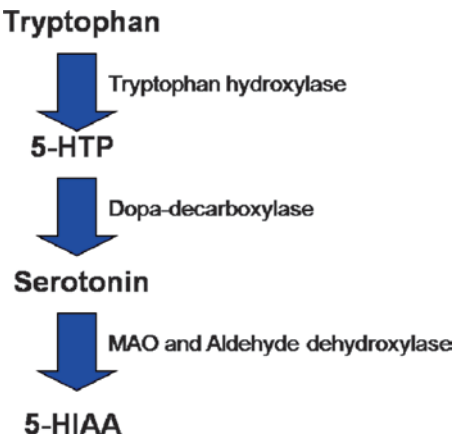

Fig. 26.2 Synthesis of 5-HIAA

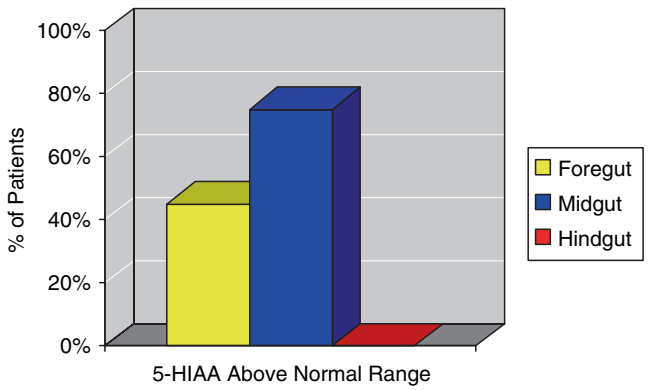

Fig. 26.3 Percentage of patients with carcinoid tumors with elevated 5-HIAA

26.2.3 Hindgut Carcinoid

This includes those tumors of the transverse colon, left colon, and rectum. They are argentaffin-negative, rarely contain serotonin, rarely secrete 5-HTP or other peptides, and usually are silent in their presentation. However, they may metastasize to the bones.

26.2.4 Bronchus

Broncho-pulmonary NETs comprise 20% of all lung cancers [7]. The biochemical findings are dependent on the histological type of bronchial neuroendocrine tumor. The typical carcinoid presents increased plasma levels of CgA. When hormone-related symptoms are present, plasma

adrenocorticotropic hormone (ACTH), growth hormone releasing hormone (GHRH), insulin-like growth factor (IGF-I), urine cortisol, urine 5-HIAA or histamine metabolites (U-methylimidazole acetic acid) may be elevated [8].

26.2.5 Thymus

The overall age-adjusted incidence of thymic carcinoids is 0.01/100,000/year. These tumors might be part of the multiple endocrine neoplasia (MEN) type 1 syndrome. According to Oberg, these tumors are very similar to bronchial carcinoids in their biochemical profile [8].

26.2.6 Stomach

There are different neuroendocrine cells in the stomach: G cells (antrum), D cells (corpus and antrum), enterochromafin-like (ECL) cells (corpus and fundus), D1 cells, enterochromafin (EC) cells, parietal cells and X cells, which have different products and are prone to tumor formation. The gastric NETs are most likely to derive from ECL cells. They constitute up to 30–40% of the neuroendocrine cells of the stomach and release histamine [9]. Gastric NETs are divided into: type 1 (multiple, small, relatively non-aggressive tumors, associated with achlorhydria – sometimes in the presence of pernicious anemia– and high gastrin levels), type 2 (associated with high levels of gastric acid and gastrin: Zollinger–Ellison Syndrome, they are larger and more prone to metastasize than type 1 tumors) and type 3 tumors (the largest gastric NET with the highest metastasis rate, are sporadic and usually present with normal gastric acid and gastrin levels) [4]. Prolonged, increased gastrin levels can produce ECL cell hyperplasia and subsequently, the development of a gastric NET [4].

With the discovery of the orexigenic gastric hormone ghrelin, it has been known that its circulating levels rise before and decrease after a meal [10].

Suppression rates of ghrelin 30–60 min after a meal in obese patients were significantly lower than those in normal-weight patients. Plasma ghrelin levels are hence elevated in diabetes. Elevated endogenous ghrelin enhances antropyloric coordination, which accelerates gastric emptying in the early stages of diabetes [11]. Thus, the physiologic functions of this new gut hormone are being elucidated and a role in NETs anticipated.

Ghrelin-immunoreactive cells are present not only in normal human gastric oxyntic mucosa, but also in all types of ECL cell NETs. Despite the frequent occurrence of ghrelin-immunoreactive cells in both the neoplastic parenchyma and the oxyntic mucosa, plasma total ghrelin concentrations do not increase above the reference range and can therefore not be used as a clinical marker to identify ghrelin-expressing ECL cells, NETs or ghrelin cell hyperplasia [12] (Fig. 26.4).

Thus, there are three different biochemical markers useful for gastric NETs: fasting serum gastrin (FSG) levels, which will be elevated in type 1 and 2 gastric neuroendocrine tumors. Of great importance is to stop the use of proton pump inhibitors (PPIs), if possible, 7 days before the test to avoid false positive results [4]. Histamine: the main secretory product of the ECL cells; and ghrelin, which have been shown to be of limited value as serum markers.

26.2.7 Pancreas

Neuroendocrine tumors of the pancreas are frequently (40–50%) non-functioning or secrete peptides with low biological impact such as pancreatic polypeptide (PP) or neurotensin. The functioning tumors are named according to their secretory product: insulinoma, gastrinoma, VIPoma, glucagonoma and somatostatinoma. The first two (insulinomas and gastrinomas) are the most frequent functioning pancreatic NETs [1].

There are six criteria for the diagnosis of insulinomas: documented blood glucose levels ≤2.2 mmol/l (≤40 mg/dl); concomitant insulin levels ≥6 μU/ml (≥36 pmol/l; ≥3 μU/ml by ICMA); C-peptide levels ≥200 pmol/l; proinsulin levels ≥5 pmol/l.; ß-hydroxybutyrate levels ≤2.7 mmol/l and absence of sulfonylurea (metabolites) in the plasma and/or urine. Further, controlled testing includes the 72-h fast, which is the *gold standard* for establishing the diagnosis of insulinoma [13]. Actually, 98% of patients with insulinomas will develop symptomatic hypoglycemia within 72 h [1]. When the patient develops symptoms and the blood glucose levels are ≤2.2 mmol/l (≤40 mg/dl), blood should also be drawn for C-peptide, proinsulin and insulin. Failure of appropriate insulin suppression in the presence of hypoglycemia substantiates an autonomously secreting insulinoma [13]. It has been proposed that the sensitivity of the 48-h fasting test is between 94.5 and 95.7% and should be enough for diagnosis of insulinoma instead of the 72-h fast test [14, 15]. In case of suspected insulinoma, it is important to keep in mind the possible differential diagnosis: nesidioblastosis, NIPHS (see discussion later) and multiple adenomas.

For gastrinomas (Zollinger–Ellison Syndrome), two measurements are critical: fasting serum gastrin (FSG) and basal gastric acid output. FSG alone is not enough because of its lack of specificity, making it impossible to distinguish hypergastrinemia due to a gastrinoma from that due to achlorhydric states. For these measures a wash-out period from PPIs treatment of 1 week is recommended [16]. If the FSG is ≥1000 ng/L (pg/ml) and the gastric pH is <2.5, the diagnosis is established [1], if the patient is normocalcemic, free of pyloric obstruction and has normal renal function [17], the

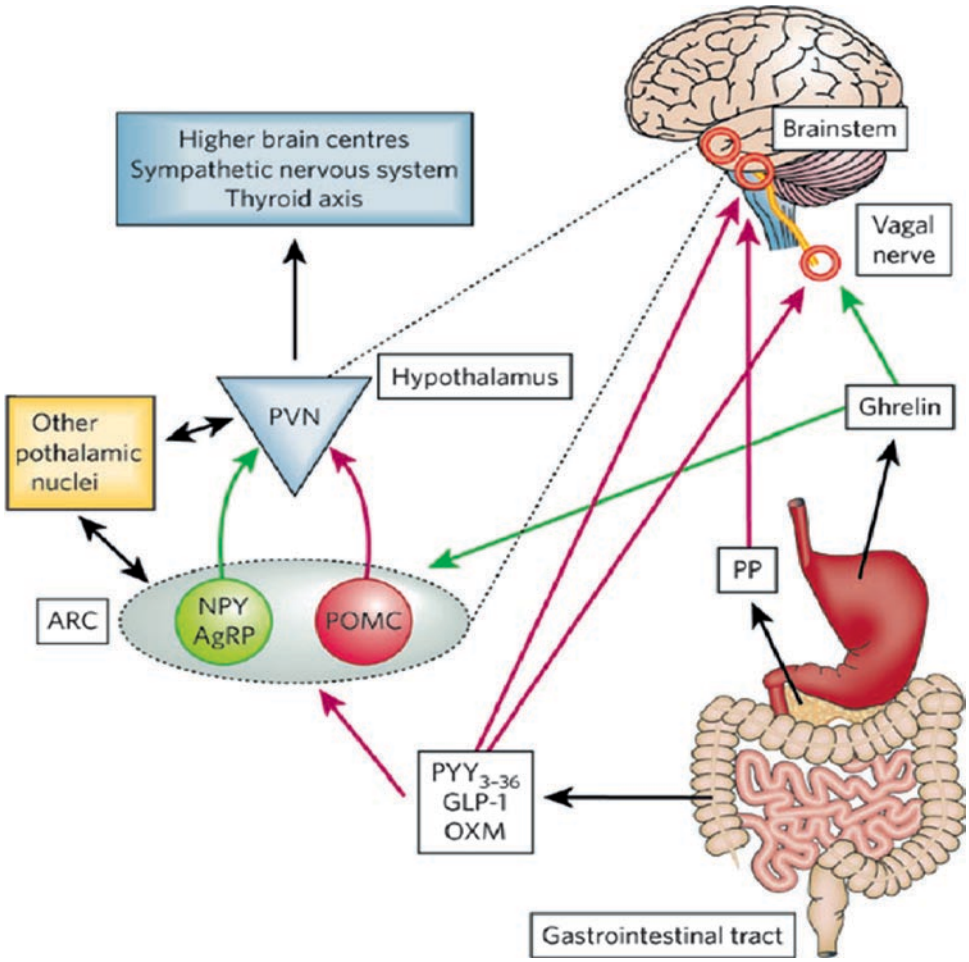

Fig. 26.4 Gut hormones and the regulation of energy homeostasis

2006 European Neuroendocrine Tumor Society (ENETS) guidelines had cutoff values of >10-fold elevation for fasting serum gastrin (FSG) and gastric pH ≤ 2 [16]. In case that the FSG values are not high enough to make a definitive diagnosis then a provocative test should be done. Administration of secretin after an overnight fast, serum for estimation of gastrin levels are collected fasting and 2, 5, 10, 15, and 30 min after the secretin bolus. In healthy people, the increase in gastrin is not higher than 50% over the baseline level; in the presence of a gastrinoma the increase is greater than 100 ng/L above the baseline levels, which will also distinguish patients with hypergastrinemia from achlorhydric states (ie: type 1 gastric NETs, use of PPIs, pernicious anemia, atrophic gastritis), who do not respond to the administration of secretin, unlike patients with a gastrinoma [4].

For VIPomas, glucagonomas, somatostatinomas and PPomas, the biochemical markers are VIP, glucagon, somatostatin and PP, respectively [1]. For every pancreatic NET, always screen for MEN type 1 syndrome measuring ionized calcium, serum parathyroid hormone (PTH) and prolactin [18]. Biochemical screening for pancreatic NETs, in the presence of suspected MEN 1 syndrome, should include; gastrin, insulin/proinsulin, PP, glucagon and CgA together have a sensitivity of approximately 70%, which can be increased if α and ß-hCG subunits, VIP and postprandial gastrin and PP measurements are added [17].

26.2.8 Duodenum

Five types of duodenal neuroendocrine tumors can currently be distinguished:

Duodenal gastrinomas are either sporadic or associated with MEN1 and are combined with a ZE syndrome. In both situations, these gastrinomas are usually not bigger than 1 cm, and are located predominantly in the upper part of the duodenum. If associated with MEN1, they are usually multiple, in contrast to sporadic gastrinomas.

Duodenal somatostatin-producing tumors account for approximately 15% of all duodenal NETs. Their preferential localization is in the region of the papilla of Vater or periampullary. They are not associated with any hormonal syndrome, but often occur in patients with neurofibromatosis type 1.

In this situation, a bilateral pheochromocytoma may occur simultaneously.

Non-functioning duodenal NETs usually consist of serotonin-producing cells. Occasionally, there are also tumors with gastrin- or calcitonin-positive cells. The prognosis of this group of non-functioning tumors is much more favorable than ZES-associated gastrinomas or ampullary somatostatin-producing tumors. Metastases are not to be expected until the tumor extends beyond the submucosa.

Poorly differentiated duodenal carcinomas occur primarily in the region of the papilla of Vater. They are hormonally inactive. At the time of diagnosis, advanced metastasis into the regional lymph nodes and the liver has usually occurred.

Duodenal gangliocytic paragangliomas occur in the vicinity of the papilla of Vater. Although the tumors are often >2 cm and infiltrate into the muscularis propria, they generally follow a benign course [19]. Thus, the most common products of duodenal NETs are gastrin and somatostatin and these are the markers considered for diagnosis.

26.2.9 Small Bowel

These are the most frequent NETs, especially appendiceal tumors. The majority is well differentiated and grows slowly. Rarely, they are less differentiated with fast growth and poor prognosis. Symptoms are atypical, diagnosis is often accidental. In 4–10% of patients, typical symptoms of carcinoid syndrome are present. The biochemical markers that should be measured in these patients are Chromogranin A and urinary excretion of 5-HIAA, which is used for the diagnosis and monitoring of the disease [20].

26.2.10 Colon and Rectum

Most of the tumors from the distal colon and rectum are nonfunctioning hindgut carcinoids. It is possible to measure peptide YY (PYY), which is a naturally occurring gut hormone with mostly inhibitory actions on multiple tissue targets which have been identified in several carcinoid tumors; a decreased expression of PYY may be relevant to the development and progression of colon adenocarcinoma [21].

26.2.11 Pheochromocytoma

Is a rare catecholamine-producing tumor usually localized in the adrenal gland that arises from neuroendocrine chromaffin cells of the adrenal medulla. Guller et al. published in 2006 that the tests of choice to establish the diagnosis of pheochromocytomas are urinary normetanephrine and platelet norepinephrine with sensitivities of 96.9 and 93.8% , respectively [22]. In a study conducted in Switzerland by Giovanella et al. in 2006, plasma metanephrines and CgA showed 95% sensitivity with comparable high specificity and diagnostic accuracy (96% and 96% for CgA, 94% and 95% for MN, respectively). If both were used then sensitivity increased to 100%. The difference found between these two markers is that only CgA was correlated with tumor mass [23]. In 2008, Bilek et al. also studied the use of CgA for pheochromocytoma, and found that it is a great marker for following response to treatment and that the levels of CgA were correlated with the size and the malignancy of the tumor [24].

26.2.12 Paragangliomas

Paragangliomas are NETs that arise from the paravertebral axis. Sympathetic paragangliomas usually hypersecrete catecholamines and are localized in the thorax, abdomen or pelvis. Parasympathetic paragangliomas are nonsecretory tumors usually localized in the head and neck area [25].

Diagnosis of paragangliomas is similar to that of pheochromocytomas since these two entities only differ in their place of origin, extra adrenal versus adrenal, respectively. Algeciras-Schimnich et al. suggested that when plasma fractionated metanephrines are measured and values are not fourfold above upper normal limit then serum or plasma CgA and urine fractionated metanephrines should be measured to confirm the diagnosis [26]. After surgery, the biochemical followup should be done 1–2 weeks later with 24-h urine fractionated catecholamines and metanephrines, if normal, complete resection is claimed, but if it persists elevated a second primary or occult metastasis should be suspected and investigated. Young et al. also proposed an annual biochemical testing followup for life, with 24-h urinary excretion of fractionated catecholamines and metanephrines or plasma-fractionated metanephrines, and only in the case of elevated levels then imaging followup is considered [27].

All patients with paragangliomas should undergo genetic testing with VHL, RET, NF1, SDHD, SDHB, and SDHC genes [25]. If positive then first-degree relatives genetic testing should be suggested and genetic counseling should be offered. First-degree relatives should always undergo biochemical testing with 24-h urine fractionated metanephrines and catecholamines [27].

26.2.13 Medullary Carcinoma of the Thyroid (MCT)

These originate from the parafollicular cells of the thyroid, which secrete calcitonin. They represent 4–10% of all

thyroid neoplasms [28]. MCT can present as two different forms, sporadic (75%) or inherited (25%) and the latter can be either isolated or part of the MEN 2 syndrome [29]. A germline autosomal dominant mutation in the RET protooncogene, which encodes for a transmembrane tyrosine kinase receptor, predisposes individuals to develop MCT. Screening for RET germline mutations has allowed for early and accurate diagnosis of patients at risk of developing MCT [30, 31].

The most common clinical presentation of MCT is a thyroid nodule, either single or as multinodular goitre. Usually, no other manifestations are present unless the tumor is already in stage IV (metastatic disease), when diarrhea and/or flushing can be present [32].

The calcitonin-secreting nature of these tumors and the fact that calcitonin is almost exclusively secreted by C-cells, explains why this hormone is the preferred biochemical marker for the diagnosis and followup of this disease; besides, it has been shown that calcitonin measurement is more sensitive than fine-needle aspiration for the diagnosis of MCT [32]. A 10-year survival of only 50% for MTC patients is reported in several series. The only possible means to improve the cure and survival rate of these patients consists in early diagnosis and early surgical treatment while the MTC is still intrathyroid [32]. Costante et al. reported in 2007 that the positive predictive value (PPV) of basal calcitonin levels over 100 pg/ml is 100% for MCT and if pentagastrin stimulation test is used, calcitonin levels above 100 pg/ml had a positive predictive value of 40%, but, below this cut-off value the false positive results increase until the PPV of basal calcitonin level >20 pg/ml is less than 25% [28]. Cohen et al. found that calcitonin levels are not only useful as diagnostic marker but they are also correlated with tumor size and metastasis, which gives some prognostic value to this hormone. When levels are less than 50 pg/ml preoperatively, the normalization of calcitonin levels postoperatively is found in 97.8% of the patients [29]. Scheuba et al. recently published that values of basal calcitonin >64 pg/ml or stimulated calcitonin levels >560 pg/ml, had a sensitivity of 100% for MCT [33]. Calcitonin increase can be observed also in parafollicular C-Cell Hyperplasia (CCH) and other extra-thyroidal conditions. The pentagastrin test is used to distinguish MCT from CCH because it is thought that the response to this stimulus is typical of pathological thyroid C cells. The cut-off value of calcitonin response between patients with MTC and CCH remains to be established [34]. Pentagastrin stimulation test is no longer available in the US but it consists on the IV injection of 0.5 μg/Kg body weight of pentagastrin and measurements of calcitonin at 0, 1, 2, 5, and 10 min after the injection; healthy people do not experience an increase in calcitonin above 200 pg/ml after the administration of pentagastrin [4]. Instead, a stimulation test can also be done with intravenous calcium infusion.

26.2.14 C-Cell Hyperplasia

This entity has been proposed to be a pre-cancerous lesion that eventually transforms into MCT. Schley, Shin, Perry and Vinik submitted a study where three cases are reported in which patients presented with flushing, abdominal pain, diarrhea and facial telangiectasia, resembling carcinoid syndrome, but the only biochemical abnormalities were elevated calcitonin levels and positive pentagastrin and calcium infusion tests. Venous sampling was performed and it localized the overproduction of calcitonin to the thyroid and histology showed C-Cell hyperplasia. After thyroidectomy, symptoms resolved and calcitonin levels too returned to normal. They proposed that the condition might be a gene mutation but so far the site has not been identified considering that RET proto-oncogene was negative in the three patients. These findings suggest that every case of flushing and diarrhea should have a calcitonin measurement, considering CCH or MCT in the differential diagnosis [35].

26.2.15 Multiple Endocrine Neoplasia (MEN) Syndromes

This entity is classified as either MEN type 1 or type 2. They are both inherited in an autosomal dominant pattern. Mutations on the MEN 1 tumor-supressor gene (inactivated) or the RET proto-oncogene (activated) are found in MEN type 1 and type 2, respectively [36].

– MEN type 1: Is characterized by hyperplasia and/or neoplasm of the parathyroid glands, entero-pancreatic NETs, and pituitary adenomas. Some patients do not present with all these tumors and hence it has been agreed that diagnosis is made when a patient presents with two of these concomitantly. To diagnose familial MEN-1 syndrome, a first-degree relative has to present at least one of the tumors mentioned above [37]. Hyperparathyroidism occurs in about 90% of patients; endocrine pancreatic tumors in 60% of patients, usually they are small and non-functional, and the most common hormonally active ones are insulinomas or gastrinomas. Pituitary adenomas are present in 40% of patients and in 60% of the patients, skin manifestations can also be present [37, 38]. Genetic studies are available for MEN type 1 syndrome; MEN 1 germline mutations are found in these patients, but its presence does not prompt any immediate intervention [39]. Piecha et al. proposed a recommendation for carriers of MEN-1 mutation to be screened biochemically every 1–3 years for hyperparathyroidism, prolactinoma, gastrinoma, insulinoma and other

– entero-pancreatic tumors [37].
– MEN type 2: This syndrome is sub-classified into type 2A, 2B and familial medullary carcinoma of the thyroid, all sharing the presence of MCT; and they are all characterized by an activating germline mutation in the RET proto-oncogene, specific for each type and which can be identified in almost 100% of the patients, with genetic testing. Once the genetic test demonstrates the mutation, a total thyroidectomy is mandatory either prophylactically in carriers or as treatment in patients who already present with manifestations of the syndrome [40]. MEN type 2A presents with MCT, bilateral pheochromocytomas and primary hyperparathyroidism, lately it has been published that Hirschprung disease could also be a manifestation of this syndrome and genetic screening for RET proto-oncogen mutation is recommended in this patients [41]; MEN type 2B is an association of MCT, pheochromocytomas and mucosal neuromas [42], these patients usually present with a marfanoid phenotype.

The biochemical studies recommended for these syndromes are the same as previously proposed for each tumor type, depending on the clinical syndrome; and in the case when MEN syndrome is suspected, genetic testing should also be performed in the patient and if positive, first-degree relatives should also be tested.

26.3 Specific Biochemical Markers for Each Clinical Syndrome

26.3.1 Flushing

26.3.1.1 Foregut

The flushing in foregut carcinoid tumors is dry, longlasting , intense and purplish or violet in contrast to the common red/pink seen in other NE-related flushing. It is related to telangiectasia and skin hypertrophy mostly in the face and upper neck but can also involve the limbs, and it can lead to a leonine appearance after repeated episodes.

26.3.1.2 Midgut

In midgut tumor, it is faint pink to red color and involves the face and upper trunk as far as the nipple line. The flush is initially provoked by alcohol and food containing tyramines (e.g., blue cheese, chocolate, red sausage, and red wine). With time, the flush may occur spontaneously and without provocation. It usually is ephemeral, lasting only a few minutes, and may occur many times per day but generally does not leave permanent discoloration.

Differential diagnosis of flushing includes the postmenopausal state, simultaneous ingestion of chlorpropamide and alcohol, panic attacks, medullary carcinoma of the thyroid, autoimmune epilepsy, autonomic neuropathy and mastocytosis [43]. In order to differentiate all those causes from a carcinoid tumor, besides knowing the differences in the characteristics of the flushing it is also necessary to know what is producing the flushing (Fig. 26.5 and Table 26.3).

Flushing in carcinoid syndrome has been ascribed to prostaglandins, kinins, and serotonin (5-HT). With the advent of sophisticated radioimmunoassay methods and region-specific antisera, a number of neurohumors now are thought to be secreted by carcinoid tumors, including serotonin, dopamine, histamine, 5-HIAA, kallikrein, SP, neurotensin, motilin, SRIF, VIP, prostaglandins, neuropeptide K, and gastrin-releasing peptide (GRP).

Feldman and O'Dorisio have previously reported the incidence of elevated levels of plasma neuropeptide concentrations. Despite the elevated basal concentrations of SP and neurotensin, these authors were able to document further increases in these neuropeptides during ethanol-induced facial flushing. We do support this contention and hasten to add that neuropeptide abnormalities frequently occur in patients with other forms of flushing and they may be of pathogenetic significance.

Several provocative tests have been developed to identify the cause of flushing in carcinoid syndrome. These tests are based upon the need to distinguish the flushing from that found in a host of other conditions particularly in panic syndrome in which the associate anxiety and phobias usually establish the cause but frequently the physician and patient need reassurance that there is *no* underlying malignancy.

Ahlman and colleagues reported the results of pentagastrin (PG) provocation in 16 patients with midgut carcinoid tumors and hepatic metastases. All patients tested had elevated urinary 5-HIAA levels, and 12 had profuse diarrhea requiring medication. PG uniformly induced facial flushing and GI symptoms in patients with liver metastases, but it had no effect on healthy, in-control patients. All patients with PG-induced GI symptoms demonstrated elevated serotonin levels in peripheral blood. Administration of a serotonin-receptor

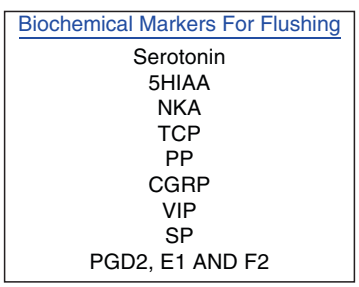

Fig. 26.5 Biochemical markers of flushing

Table 26.3 Differential diagnosis of flushing and recommended tests

Clinical condition	Tests
Carcinoid	Serotonin, 5HIAA, NKA, TCP, PP, CGRP, VIP, SP, PGD2, PGE1, PGF2
Medullary carcinoma of the thyroid	Calcitonin, Ca2+ Infusion, RET proto-oncogen
Pheocromocytoma	CgA, Plasma free metanephrines, urine metanephrines, VMA, Epi, Norepi, Glucagon Stimulation, MIBG
Diabetic autonomic neuropathy	HRV, 2hs PP Glucose
Menopause	FSH
Epilepsy	EEG
Panic attack	Pentagastrin, ACTH
Mastocytosis	Plasma Histamine, Urine Tryptase
Hipomastia and mitral valve prolapse	Cardiac Echo

antagonist had no effect on serotonin release but it completely aborted the GI symptoms. The authors emphasized the improved reliability of PG compared with calcium infusion, another provocative test popularized by Kaplan and colleagues, and pointed out that PG provocation occasionally can be falsely negative in patients with subclinical disease. Our own experience is that PG uniformly induced flushing in patients with gastric carcinoid tumors that was associated with a rise in circulating levels of SP in 80%. Thus, SP is one neurohumor that may be involved in the flushing of carcinoid syndrome.

Substance P has been found in tumor extracts and plasma from patients with carcinoid tumors and, in one reported case, was useful for tumor localization. Neurokinin A, its amino-terminally extended form, neuropeptide K, and SP are a group of peptides (i.e., tachykinins) with common biologic properties. Norheim and colleagues measured peptide responses to PG or ingestion of food or alcohol in 16 patients with metastatic carcinoid tumors and demonstrated twofold or greater increases in neurokinin A and neuropeptide K in 75% of patients, as well as variable increases in SP in approximately 20% of patients.

Conlon and colleagues used region-specific antisera to SP and neurokinin A to measure circulating tachykinins during a meal-induced flush in 10 patients with metastatic carcinoid tumors. Five patients had undetectable levels of neurokinin A and SP after stimulation, thus suggesting that elevated tachykinin concentrations are not a constant feature of such patients. The authors also studied the effect of a somatostatin-analogue administration on meal-induced tachykinin responses in three patients with carcinoid tumors. Flushing was aborted in two patients, but tachykinin levels were only partially suppressed, indicating that these peptides cannot be solely responsible for the carcinoid flush. When the diagnosis of the underlying cause of flushing has been established, pathogenesis-oriented treatment can be very helpful [43].

Janet et al. also performed a study, in which they used patients with metastasizing ileocecal serotonin-producing carcinoid tumors (MISPCs) and looked for the relationship of flushing to tachykinin production. They concluded that MISPCs produce many biologically active substances with partially overlapping biological functions. The biological processes underlying the specific symptoms of the carcinoid syndrome are probably multifactorial. In their study, they confirmed results from earlier studies showing that tachykinins and 5-HIAA levels are elevated in patients with daily episodes of flushing. The hormone effects were not mutually independent. It is possible that the development of flushing is the result of multi-hormonal stimulation. Other biologically active substances, such as kalikrein, and prostaglandins, may also contribute [44].

26.3.2 Diarrhea

Secretory diarrhea is characteristic of NETs, causing large volume stools, persists with fasting and there is no osmotic gap between serum and stool (Fig. 26.6). There are several causes of secretory diarrhea that need to be taken into consideration in the differential diagnosis: Watery Diarrhea, Hypokalemia, Hyperchlorhydria and Acidosis (WDHHA) syndrome, the Zollinger–Ellison (Z–E) syndrome, carcinoid tumors, MCT, secreting villous adenoma of the rectum, surreptitious laxative abuse and idiopathic.

NETs can produce diarrhea by different mechanisms depending on their secretory products. Gastrin can increase the acid secretion by the stomach, which in turn inactivates lipase, amylase and trypsin and damages the mucosa of the small bowel, leading to decreased absorption and impaired digestion in the small bowel, exceeding the absorptive

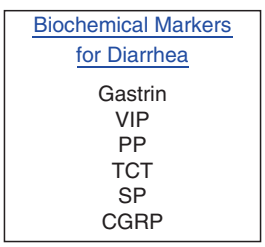

Fig. 26.6 Biochemical markers for diarrhea

capacity of the colon, what gives an increased fecal volume and malabsorptive syndromes and sometimes steatorrhea. On the other hand, carcinoid or other NETs can produce other substances like VIP, PP, SP, CGRP and/or TCT, all of which will act on the small bowel increasing the secretion of fluids and ions, which in turn will also exceed the colonic absorptive capacity producing an increased fecal volume as well as great losses of potassium and bicarbonate.

A disturbing cause that may be very difficult to differentiate is laxative abuse, and, in all circumstances, a KOH stool preparation to detect laxatives is mandatory. Measurement of intestinal secretion by passing a multilumen tube and quantifying electrolytes and water transport, in addition to the measurement of stool electrolytes, which should account for the total osmolarity, will help to exclude laxative abuse.

It is important to mention that Janet et al. found, in their study of tachykinins and neuroendocrine tumors, that there is an association between the elevation of tachykinins and the severity of the diarrhea. They concluded that all biochemical markers concentrations were elevated in patients with daily episodes of diarrhea, though, the association between increased plasma Tachykinins and the severity of diarrhea was independent of both CgA and 5-HIAA concentrations [44].

26.3.3 Bronchoconstriction

Wheezing due to bronchospasm occurs in one-third of patients with carcinoid syndrome. Lung function tests show a prolonged forced expiratory volume in the first second (FEV1). Differential diagnosis are asthma and chronic obstructive pulmonary disease. In the carcinoid syndrome, the cause of bronchoconstriction is usually substance P, histamine or serotonin that should be measured in patients who present with this symptom [4].

26.3.4 Dyspepsia or Peptic Ulcer

The Zollinger–Ellison syndrome is characterized by peptic ulcers and diarrhea that responds to therapy with proton pump inhibitors (PPIs), in the setting of hypergastrinemia and low gastric pH. Gastrinomas are localized 90% of the time in the "gastrinoma triangle" (Fig. 26.7). As discussed in the previous section, the measurements that should be drawn for these tumors are FSG and gastric acid output.

26.3.5 Hypoglycemia

The Whipple's Triad (symptoms of hypoglycemia, low blood glucose levels <40 mg/dl; and relief of symptoms with glucose)

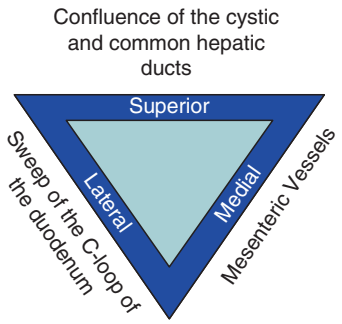

Fig. 26.7 Gastrinoma triangle

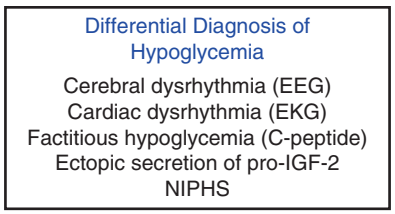

Fig. 26.8 Differential diagnosis of hypoglycemia

is the clinical presentation of insulinomas but other causes should be ruled out (Fig. 26.8).

Patients with non-insulinoma pancreatogenous hypoglycemia (NIPHS) present with postprandial neuroglycopaenia symptoms (within 4-h of meal ingestion), have negative 72-h fasting test, negative tumor localization studies and on histological diagnosis, hypertrophy or nesidioblastosis rather than an insulinoma are found [17, 45]. Other possible causes that should be thought of are: fasting, autoimmune (insulin antibodies), counter regulatory hormone deficiency, drug-induced and factitious hypoglycemia. In order to exclude all the other causes, clinical suspicion together with measurement of hormones or peptides should be used.

In the case of hypoglycemia the recommended biochemical markers are insulin, IGF-2, C-peptide, glucagon-like peptide type 1 (GLP-1), GIP, sulfonylurea, ACTH, GH, insulin antibodies and liver enzymes [4].

26.3.6 Dumping Syndrome

This manifestation occurs after surgery when the pylorus has been resected or inactivated. It can be early, when symptoms resemble shock, or late which presents as hypoglycemia. For the diagnosis of this syndrome a provocative test is done giving the patient a high-calorie, carbohydrate-rich breakfast with 750 kcal (21 g protein, 30 g fat and 99 g carbohydrate) that should be ingested in 10 min to produce the maximum response. After completion of the meal, blood sample is collected at 10, 15, 30, 45, 60, 120 and 180 min; to measure

glucose, insulin, C-peptide, motilin, PP and GLP-1 levels [4]. An exaggerated insulin and GLP-1 response to the meal is found in gastric bypass patients with the syndrome although the case and relationship between the hormonal overproduction and the clinical syndrome remain controversial.

26.3.7 Pellagra

Caused by the deficiency of Niacin, due to the detour of the tryptophan pathway towards the production of increased amounts of serotonin (Fig. 26.9).

26.3.8 Glucagonoma or the "Sweet" Syndrome

Diabetes accompanied by the 4D syndrome (Dermatosis: Necrolytic migratory erythema, Depression, Deep venous thrombosis and Diarrhea), is the clinical presentation of glucagonomas.

Glucose intolerance in the glucagonoma syndrome may relate to tumor size. Fasting plasma glucagon levels tend to be higher in patients with large hepatic metastases than in those without hepatic metastases [47], and all patients with large hepatic metastases have glucose intolerance. Massive hepatic metastases may decrease the ability of the liver to metabolize splanchnic glucagon, thus increasing peripheral plasma glucagon levels. Glucagon may not directly induce hyperglycemia, however, unless metabolism of glucose by the liver is directly compromised. Another factor may be variation in the molecular species of glucagon that is present in each case and its biologic potency [48].

In previously reported cases of glucagonoma in which plasma glucagon concentrations were measured by radioimmunoassay, fasting plasma glucagon concentrations were 2100 ± 334 pg/mL. These levels are markedly higher than those reported in normal, fasting subjects (i.e., 150 pg/mL) or in those with other disorders causing hyperglucagonemia, including diabetes mellitus, burn injury, acute trauma, bacteremia, cirrhosis, renal failure, or Cushing's syndrome, where fasting plasma glucagon concentrations often are elevated but less than 500 pg/mL.

As with other islet cell neoplasms, glucagonomas may overproduce multiple hormones like insulin, ACTH, PP, PTH or substances with parathyroid hormone-like activity, gastrin, serotonin, VIP, and melanocyte-stimulating hormone (MSH), in that order of frequency [49].

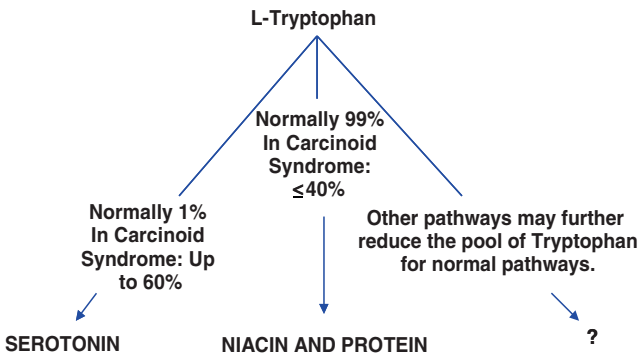

Fig. 26.9 Serotonin and Niacin synthesis in normal conditions and in the carcinoid syndrome [46]

26.3.9 Acromegaly or Gigantism

Can present when any NET secretes GH or GHRH. Basal levels of GH and IGF-1 are usually enough to make a diagnosis; but in 15–20% of the patients further investigation is needed to show non-suppressibility of GH to an oral glucose tolerance test (OGTT), a somatostatin inhibition test or a bromocryptine-suppression test. In the case of the OGTT also, measure lipids and insulin, which should also be suppressed. Other pituitary and hypothalamic hormones should also be measured, such as prolactin, the α and ß-subunits of gonadotropins and thyroid-stimulating hormone (TSH) [4].

26.3.10 Cushing's Syndrome

A pituitary tumor, small cell carcinoma of the lung (known to produce ACTH) or an ACTH-secreting NET will present clinically as the Cushing syndrome from oversecretion of cortisol, adrenal androgens and 11-deoxycorticosterone. To reach the diagnosis, several steps should be followed. New guidelines for the diagnosis of Cushing's syndrome have been published, though some of the recommendations are based on low quality evidence. Their proposed approach is as follows:

After excluding exogenous glucocorticoid use (iatrogenic Cushing's syndrome) patients with unusual features for age like osteoporosis or hypertension, patients with multiple and progressive features predictive of Cushing's syndrome (easy bruising, facial plethora, proximal myopathy or muscle weakness, reddish/purple striae, weight gain in children with decreasing growth velocity) and patients with adrenal incidentaloma compatible with adenoma should undergo testing for Cushing's syndrome starting with one test with high diagnostic accuracy: Urine free cortisol (at least two measurements), late night salivary cortisol (two measurements), 1 mg overnight dexamethasone suppression test (DST) or

longer low-dose DST (2 mg/d for 48 h). If the test is negative and the pretest probability was low then followup in 6 months is recommended if progression of symptoms; in case of a negative test but with a high pretest probability then more than 1 test should be performed. In some cases, a serum midnight cortisol or dexamethasone-CRH test should be done [50].

26.4 Classification of the Biochemical Markers According to Their Use

26.4.1 Diagnostic

26.4.1.1 Chromogranin A (CgA) and Chromogranin B (CgB)

Both are part of the granin family. They are stored and secreted from vesicles present in the neuroendocrine cells, together with other peptides, amines and neurotransmitters [51]. CgA is the best studied [52] and most used (Fig. 26.10). But CgA is not perfect. As Mats Stridsberg and his colleagues reported in 2007, there are some common conditions that can increase the levels of this marker and give false positive measurements: decreased renal function and treatment with proton pump inhibitors [53] and even essential hypertension [54]; these problems are not seen with CgB, so they proposed measurement of CgB as a complement to CgA [53].

The most important characteristic of these markers is that they are not only secreted by the functional tumors but also by those less well-differentiated NETs that do not secrete known hormones [2].

CgA has been shown to be increased in 50–100% of patients with NETs [2, 55]. Tumor markers in neuroendocrine tumors(Digestion 2000, 33–8) depending on localization (gastrinomas 100%, pheochromocytomas 89%, carcinoid tumors 80%, non-functioning tumors of the endocrine pancreas 69% and medullary thyroid carcinomas 50%). In addition, blood levels depend upon tumor mass, burden or progression and malignant nature of the tumor [24, 56].

Sensitivity and specificity of CgA depends on many factors. For example, sensitivity varies from 77.8 to 84.0% and specificity from 71.3 to 85.3% depending on the assay used, and of great importance is to establish the cut-off value that gives the highest sensitivity without compromising the specificity [57]. Another utility of CgA is to discriminate between patients with or without metastasis, which also depends on the assay and the cut-off values used, with a sensitivity of 57.0–63.3% and specificity 55.6–71.4% [57].

Fig. 26.10 Primary structure of the Chromogranin A molecule showing several peptides that are derived after the enzymatic cleavage of CgA, like pancreastatin, catestatin, vasostatin; which have, biological activity and may contribute to the clinical syndrome

An important consideration is the fact that CgA is strongly correlated with tumor mass, as previously stated, and therefore, small tumors may go undetected [56].

26.4.1.2 Pancreatic Polypeptide (PP)

Is considered another non-specific biochemical marker, but, due to its low sensitivity, many physicians prefer not to use it. In a study conducted by Panzuto et al. in Rome, Italy in 2004, PP sensitivity was 54% in functioning tumors, 57% in non-functioning, 63% in pancreatic tumors and 53% in gastrointestinal tumors. Specificity was 81% compared with disease-free patients, and 67% compared to non-endocrine tumor patients. But when combined with CgA, the sensitivity increased compared to either of the markers alone. When used in combination, the sensitivity of these markers is: for gastro-entero-pancreatic neuroendocrine tumors (GEP NETs) 96%; for non-functioning tumors 95% and for pancreatic tumors 94% [58].

26.4.1.3 Neuron-Specific Enolase (NSE)

Are enzymes that occur mainly in cells of neuronal and neuroectodermal origin. NSE has been found in thyroid and prostatic carcinomas, neuroblastomas, small-cell lung carcinoma, carcinoids, GEP NETs and pheochromocytomas. Despite its high sensitivity (100%), its use is limited as a blood biochemical marker for neuroendocrine tumors due to its very low specificity (32.9%) [59].

26.4.2 Follow-up, Treatment Response and Prognosis

26.4.2.1 CgA

Other than the applications of CgA as previously discussed, this marker can be used for prognosis and followup. Jensen et al. found that a reduction on CgA levels ≥80% after cytoreductive surgery for carcinoid tumors and predict symptom relief and disease control; further,it is associated with improved patient outcomes, even after incomplete cytoreduction [60].

26.4.2.2 Pancreastatin

One of the post-translational processing products of CgA has been found to be an indicator of poor outcome when its concentration in plasma is elevated before treatment in patients with NETs. A level >500 pmol/L is an independent indicator of poor outcome. This marker is also known to correlate with the number of liver metastasis, so it would be appropriate to use it in the followup of NET patients. Furthermore, Stronge et al. found an increase in pancreastatin levels following somatostatin analogue therapy associated with a poor survival [61]. Other studies have shown that pancreastatin should be measured prior to treatment and monitored during and after it. Plasma levels of this marker above 5000 pg/ml pre-treatment were associated with increased peri-procedure mortality in patients with NETs that underwent hepatic artery chemoembolization (HACE) [62].

All these observations lead to the conclusion that pancreastatin should be a mandatory biochemical measurement in the battery of laboratory studies assessed in the NET patients, not only for diagnosis but more importantly, to monitor treatment response and predict survival.

26.4.2.3 Neurokinin A

Has been shown to have strong prognostic value. Turner et al. in 2006 showed that in patients with midgut carcinoid that have raised plasma NKA, a reduction of this biochemical marker after somatostatin analogue (SSA) therapy, was associated with an 87% survival at one year compared with 40% if it increased. They also concluded that any alteration in NKA predicts improved or worsening survival [63].

26.5 Biochemical Markers for Bone Metastasis (Figs. 26.11 and 26.12)

Metastases from NETs can be either lytic and/or osteoblastic

There may be an increased osteoclast activity contributing to lytic lesions and or an increase osteoblastic activity responsible for blastic metastases. Bone markers in lytic and

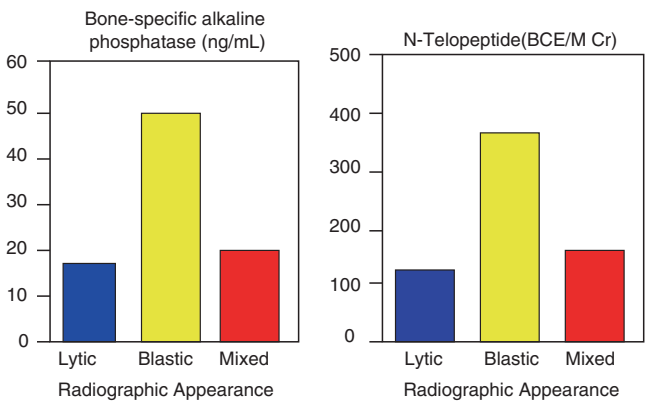

Fig. 26.11 Bone markers in patients with lytic and blastic metastases

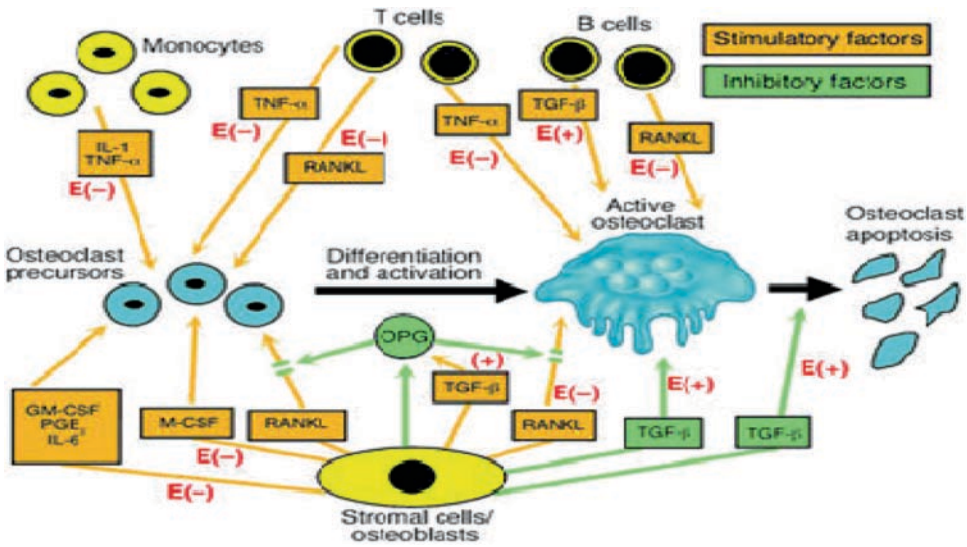

Fig. 26.12 Bone Turnover. The figure shows the factors that stimulate and the factors that inhibit the differentiation and activation of osteoclasts and hence bone resorption

osteoblastic metastases that may assist in the evaluation of stage as well as response to therapy include bone alkaline phosphatase (bAP), an indicator of osteoblast function, and urinary N-telopeptide, which reflects osteoclast activity or bone resorption. Somewhat paradoxically, only blastic metastases show an increase in both markers as indicated in Fig. 26.9 [64].

Increased osteoclast activity predicts a poor outcome, with a Relative Risk (RR) for high N-telopeptide (>100 nmol BCE/mM creatinine) of: skeletal-related events RR: 3.3 ($p<0.001$); disease progression RR: 2.0 ($p<0.001$); and death RR: 4.6 ($p<0.001$) [65].

The following markers are recommended:

Bone formation markers: Serum PINP, serum bone alkaline phosphatase (bAP), serum osteocalcin, osteoprotegrin (OPG).

Bone resorption markers: Urine N-telopeptide , serum CTX, serum N-telopeptide and serum RankL

Markers of malignancy: PTHrP in blood. Perhaps IGF-1. Calcitonin, TGF-β and endothelin-1.

Markers of cytokine excess: Serum IL-1 and IL-6.

Vitamin D metabolism: Serum 25-hydroxy vitamin D (25-OH D) and ionized calcium.

26.6 Biochemical Markers for Cardiac Involvement

Carcinoid heart disease is a unique cardiac disease associated with NETs and may be seen in up to 60% of patients with metastatic carcinoid. Valvular disease is the most common pathologic feature and tricuspid damage is found in 97% and pulmonary valve disease in 88% with 88% displaying insufficiency and 49% stenosis. The distinctive carcinoid lesion consists of deposits of fibrous tissue devoid of elastic fibers known as carcinoid plaque. The deposits are found on the endocardial surface on the ventricular aspect of the tricuspid leaflet and on the arterial aspect of the pulmonary valve cusps [66].

Although the precise cause for the plaque formation is not entirely clear, the direct actions of serotonin and bradykinin have been implicated in animal studies. This finding is corroborated by the observation that the appetite-suppressant drug fenfluramine, which releases serotonin, has been noted to cause valvular distortion similar to that seen in carcinoid heart disease [67]. Values of serotonin greater than 1000 ng/ml seem to consort with the development of carcinoid heart disease. Possibly for this reason alone, in treating these patients all attempts should be made to keep serotonin levels down in addition to relieving symptoms and slowing or abrogating tumor growth.

26.7 Octreotide Levels

Recenty it has been published that there has been a clinical escape from therapy with LAR Octreotide and that plasma octreotide levels have fallen with time from that expected based upon the dose of long-acting repeatable (LAR) octreotide. For this reason, Woltering et al. proposed adding the measurement of octreotide levels to the battery of studies done in the followup of patients with NETs who are being treated with this drug and who present with progressive symptoms, rising biomarkers or progressive growth of tumors on radiographs. When levels are less than 10,000 pg/ml, the

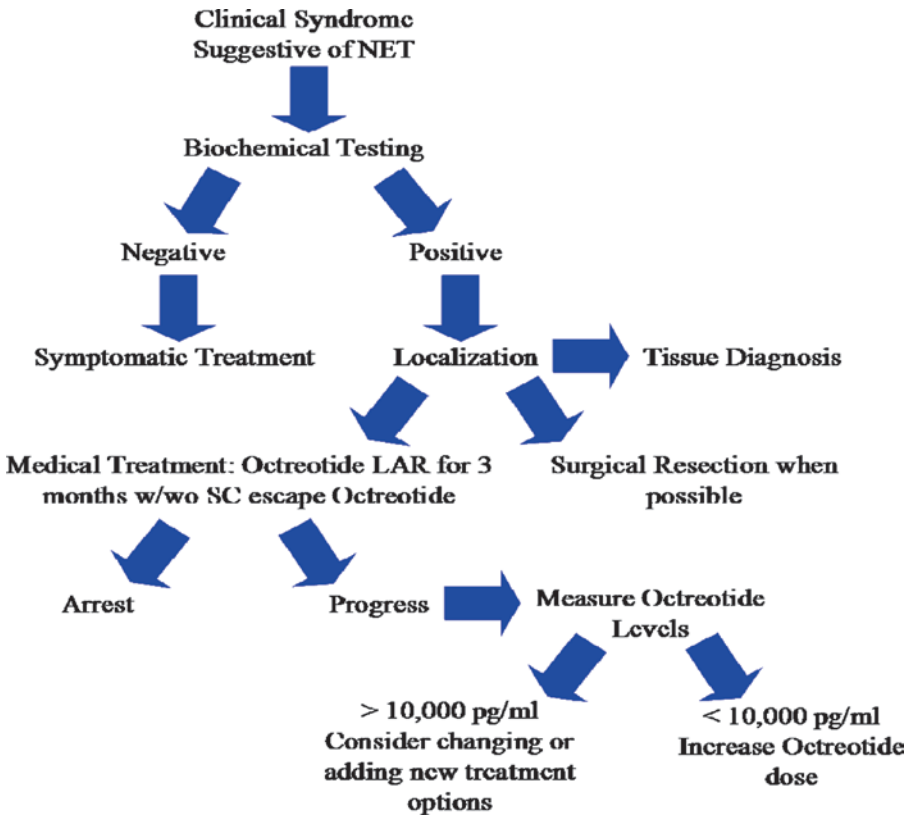

Fig. 26.13 Algorithm for diagnosis and follow-up of NETs

dose of LAR octreotide should be adjusted based on serial plasma octreotide determinations. If optimal levels (10,000–15,000 pg/mL) cannot be reached with higher LAR doses, then switching the patients to intermittent dose or continuous subcutaneous infusion octreotide therapy should be considered [68].

26.8 Summary and Conclusion

To conclude, this algorithm (Fig. 26.13) proposes a summary of the steps for diagnosis and management of NETs starting at the presentation of a suggestive clinical scenario.

It is the purpose of this chapter to show the importance of recognizing, as early as possible, the clinical syndromes that suggest a NET as one of the differential diagnosis, and once suspected, look for the appropriate biochemical markers that will confirm the diagnosis or confidently discard it.

NETs are small, slow-growing neoplasms, usually with episodic expression that makes diagnosis difficult, erroneous and often belated; for these reasons, a high index of suspicion is needed and it is important to understand the pathophysiology of each tumor to decide which biochemical markers are more useful and when they should be used.

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Chapter 27
Surgical Management of Endocrine Disorders

Joseph B. Lillegard, Travis J. McKenzie, and Geoffrey B. Thompson

27.1 Introduction

The twentieth 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 will focus on the surgical management of endocrine disorders involving the thyroid, parathyroid, adrenal, and pituitary glands, as well as the gastroenteropancreatic system, that are all subject to the development of endocrine tumors and hyperplasia.

27.2 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 US adult population. Upward of 50% of adults have demonstrable thyroid nodules when ultrasonography or autopsy studies are considered [1]. Most important, however, is that less 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 (FNA) biopsy is the most sensitive and specific preoperative indicator of thyroid malignancy. Upward of 60% of all FNAs 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 (MTC), anaplastic thyroid carcinoma, or metastases). Twenty percent of FNAs are considered suspicious either for follicular or Hurthle cell neoplasms or papillary thyroid carcinoma (PTC) [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 FNAs are indeterminate or nondiagnostic due to an inadequate or a cellular 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, and 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 rapidly correct 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 the 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.

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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 only be performed at high-volume centers after a thorough discussion of the various treatment options. In experienced hands, 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 effectively managed 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 melanoma [8]. 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 one thousand cancer-related deaths [9]. PTC and its follicular variant account for 80–90% of all primary thyroid cancers. Follicular and Hurthle 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 pre-existing, long-standing, goiter. Pathology will sometimes reveal areas of differentiated, follicular-cell-derived (FCD) cancer among the anaplastic component, 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 are dead within 12–18 months 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, PTC is among the best. Fortunately, PTC accounts for nearly 85% of all new thyroid cancers. Of these papillary cancers 80–85% can be classified as low risk, associated with a 20-year cause-specific mortality of <1% with proper treatment [12].

What is meant by low-risk PTC? Review of data, from over 2,000 patients, subjected to a multivariate analysis, at Mayo Clinic since 1945, has demonstrated that several variables can collectively determine the risk of dying from papillary thyroid cancer. These include: distant Metastases, Age (>45), Completeness of resection (yes or no), Invasion of contiguous structures (trachea, strap muscles, nerve, esophagus), and Size 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 an FNA, 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 MTC in detecting clinically occult lymph node metastases.

Ultrasound can help decide whether a central compartment node dissection alone will suffice or whether unilateral

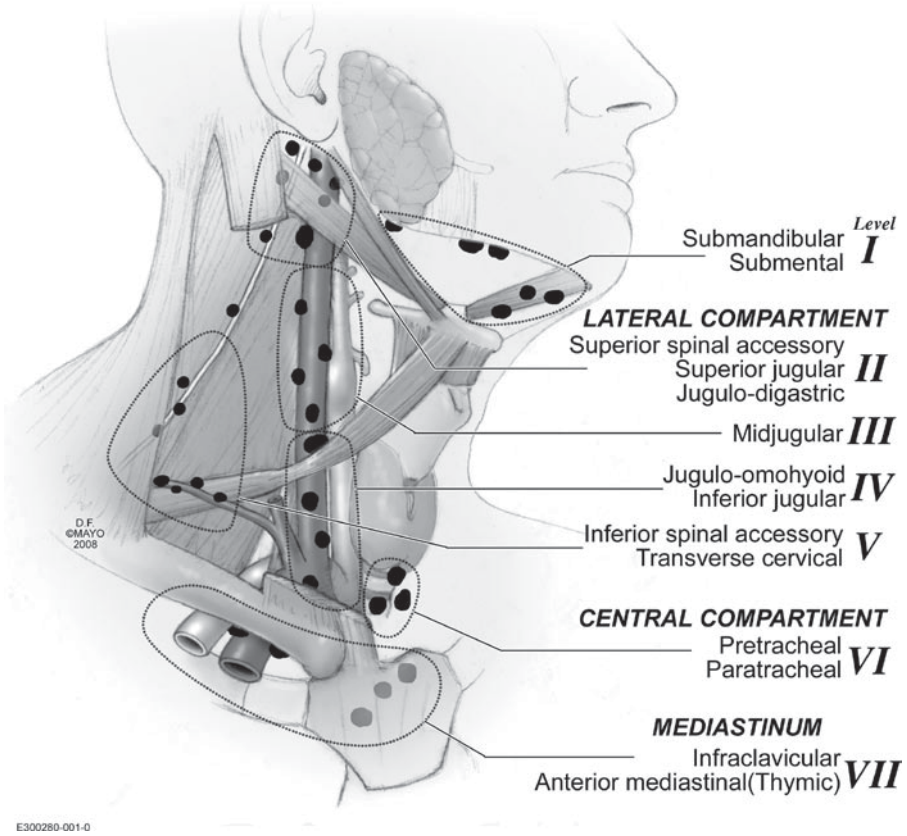


Fig. 27.1 Lymph nodes removed during a central compartment node dissection and a modified neck dissection. (Reprinted from Musholt TJ, Moley JF (1997) Management of persistent or recurrent medullary thyroid carcinoma. Prob Gen Surg 14:89–109, Lippincott, Williams & Wilkins, with permission)

or bilateral modified neck dissections are indicated (Fig. 27.1). In a recent retrospective cohort study, 702 patients underwent preoperative ultrasound for both primary and reoperative papillary thyroid cancer. Nonpalpable lymph nodes were detected in 231 (32.9%) patients, thereby altering the operative procedure performed [14]. Authors also cite that even in patients with palpable lymph nodes, ultrasound helped guide the extent of lymphadenectomy. Central compartment nodes, including 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 the sparing of the sternocleidomastoid muscle and the spinal accessory nerve.

Lymph node metastases are an interesting curiosity in low-risk PTC. 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 contralateral side, in order to reduce the risk of contralateral lobe recurrence. This is seven times more likely to occur when lobectomy alone is performed [15]. Total thyroidectomy should be performed when PTC is grossly bilobar or multicentric (30%). Central compartment nodes should always be removed and lateral nodes 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 four to six weeks' time [16].

Few 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 in order 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 since high-resolution neck ultrasonography and TSH-suppressed thyroglobulin levels will normally suffice [17].

Patients with FCD thyroid cancer may benefit from lifelong TSH suppression with thyroxine in order 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 [18].

Fifteen percent of patients with PTC 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 T_4 are essential for these high-risk patients.

The steady decline in both follicular and anaplastic thyroid cancers in the US has paralleled the decrease in endemic goiter. Most follicular and Hurthle cell neoplasms by FNA turn out to be adenomas on frozen and permanent sections. Frozen section evaluation of follicular neoplasms is highly accurate at Mayo Clinic [19] and reduces the need for second operation (completion thyroidectomy) in most cases. In a recent review of over 1000 follicular and Hurthle cell neoplasms, only 8% were found to be malignant on permanent section. Most follicular and Hurthle 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. Hurthle cell carcinoma patients should also routinely undergo a central compartment node dissection because of a 30% risk of nodal involvement. Patients with follicular carcinomas should at least have lymph nodes sampled in the central compartment in case permanent sections demonstrate the follicular variant of PTC. Lobectomy alone may be sufficient for small (<2 cm), minimally invasive follicular cancers in young people, provided the contralateral lobe is ultrasonographically normal [20]. Widely invasive and Hurthle 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. Hurthle cell cancers tend not to take up radioiodine.

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 (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-proto oncogene 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 [21]. 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, in order 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 re-exploration), 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 days. Endoscopic neck surgery may provide slight improvement in cosmesis over conventional surgery, but this has yet to be proven in controlled, scientific studies [22].

27.3 Hyperparathyroidism

Primary hyperparathyroidism (HPT) affects 1 in 1,000 men and 1 in 500 women in the US. 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. 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 [23].

Most cases of sporadic HPT involve single adenomas (85–90%). Less 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. Over 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 I 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 IIA patients less often develop HPT (<30%), and usually only one or two glands are involved. Because sequelae are less severe in MEN IIA 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 [24].

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 [25]. 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.

The traditional surgical approach to HPT has been bilateral neck exploration under general anesthesia. With the advent and refinement of minimally invasive techniques in conjunction with advances in preoperative imagining, the current indications for bilateral neck exploration are changing. The recent National Institutes of Health consensus meeting recommends that all patients should undergo preoperative localization studies in an effort to determine feasibility of minimally invasive parathyroidectomy (MIP) [26]. A meta-analysis of the sensitivity and specificity of sestamibi scanning in 6,331 cases was of 90.7% and 98.8%, respectively, and determined that 87% of the patients with sporadic HPT would be candidates for MIP (Fig. 27.2) [27]. This allows the surgeon to perform a minimal access parathyroid procedure (MAP) through a small incision (3 cm), under local or regional anesthesia in the outpatient setting (Fig. 27.3). Regional anesthesia avoids complications associated with general anesthesia, including endotracheal intubation, which has been reported to cause vocal cord changes in up to 5% of patients [28]. The dissection is limited and is directed by the sestamibi 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 false-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. In a recent series of 656 consecutive parathyroidectomies (of which 255 were minimally invasive) between 1990 and 2001, there were no significant differences in complication or cure rates. MIP was associated with an approximately 50% reduction in operating time, a sevenfold reduction in length of hospital stay, and a mean cost savings of $2,693 per procedure [29]. Some surgeons have advocated the use of an intraoperative radioguided probe, for these procedures, following the intravenous injection of sestamibi. In most centers, this methodology has proven less accurate than the procedures described earlier [30].

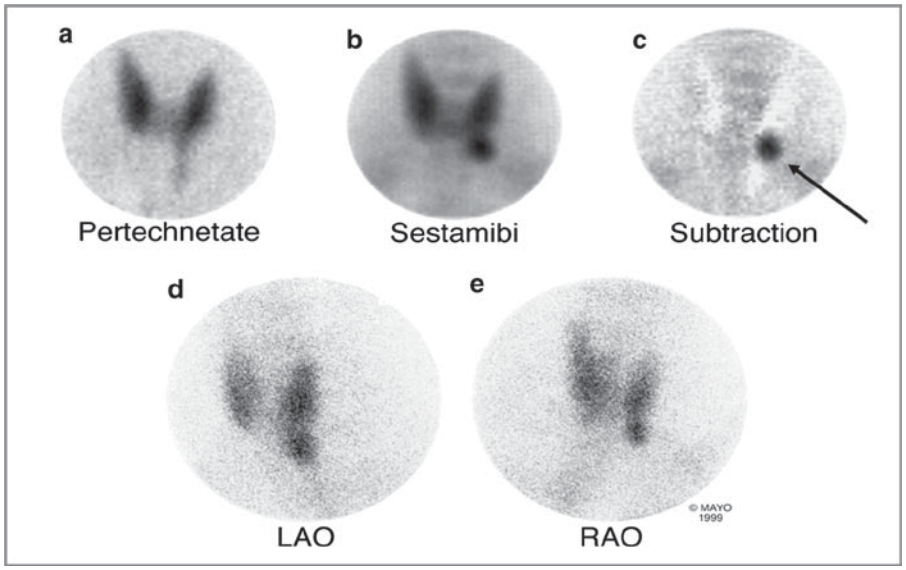


Fig. 27.2 Sestamibi parathyroid scan demonstrating a left inferior parathyroid adenoma (*arrow*)

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 [31].

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 [32].

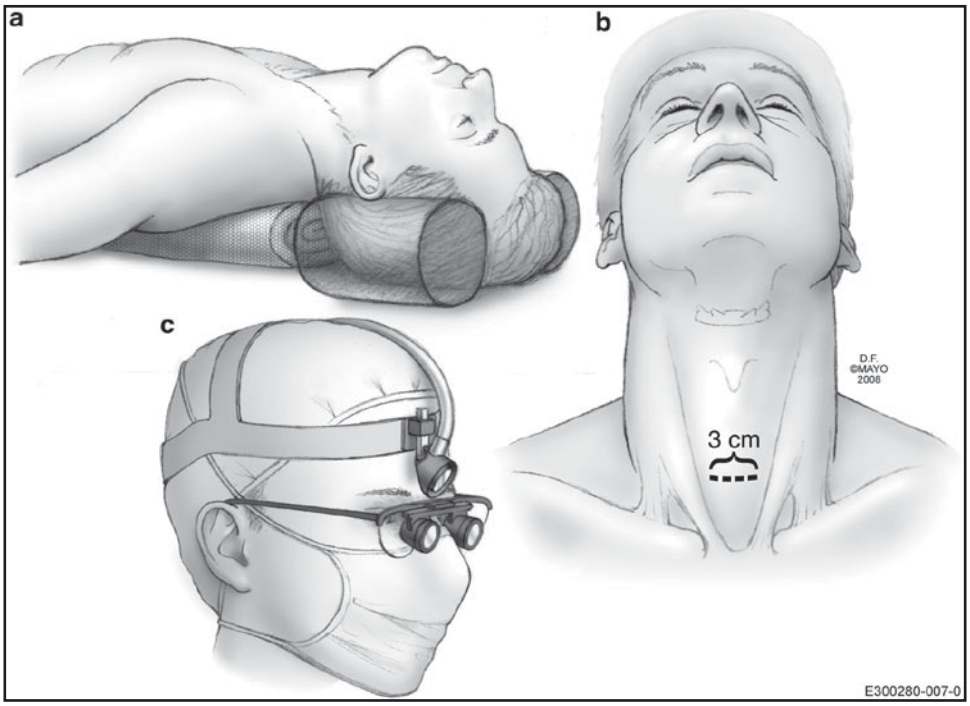
27.4 Adrenal Disorders

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 over 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 [33].

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 [34]. Laparoscopic adrenalectomy has been a tremendous advance with significant reduction in pain, hospital stay, time to return to normal, and improved cosmesis when compared to open posterior and anterior adrenalectomy (Fig. 27.4).

Pheochromocytomas occur in about 4 per 1 million individuals. Most are sporadic and unilateral, but approximately 10% occur in association with MEN II 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 extra-adrenal tumors (paragangliomas). Symptoms typically occur in the form of "spells" characterized by paroxysmal hypertension, with headaches, panic 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,



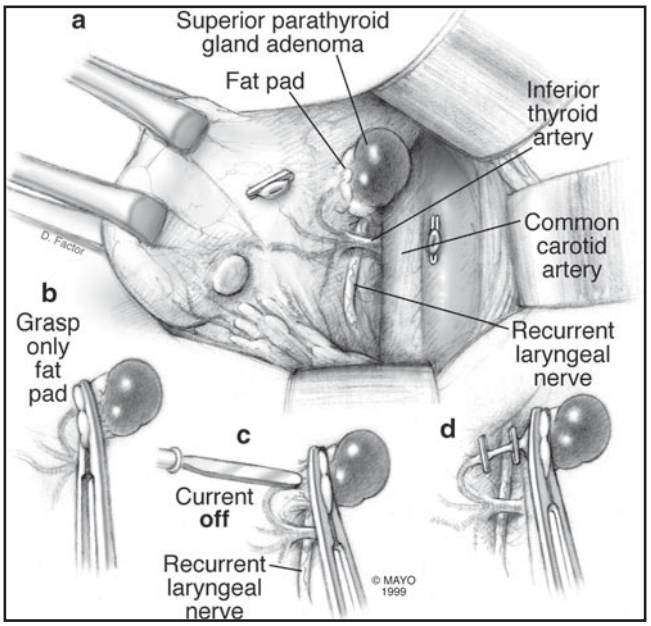


Fig. 27.3 (**a**, **b**) Minimal access parathyroid procedure with excision of left superior parathyroid adenoma. (Reprinted from Thompson GB (1999) "No frills" image-guided exploration. Oper Tech Gen Surg 1(1):34–48, W.B. Saunders Company, with permission)

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 alpha and beta 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 I^{131} MIBG or external beam radiation therapy may provide effective palliation in select patients [35].

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-third of patients. Profound muscle weakness is another manifestation secondary to the hypokalemia. The diagnosis should be suspected when high

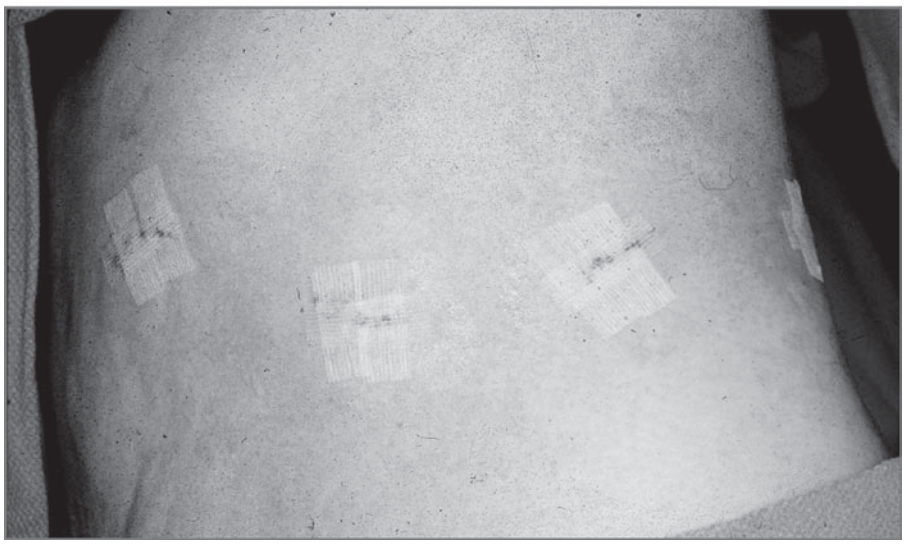


Fig. 27.4 Incisions for laparoscopic left adrenalectomy

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 a 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 [36].

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 syndrome. The inability to suppress cortisol levels with dexamethasone and undetectable adrenocorticotropic hormone (ACTH) levels confirms 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 [37].

Patients with persistent or recurrent Cushing disease following transsphenoidal surgery benefit from bilateral laparoscopic adrenalectomy. The incidence of Nelson syndrome is not as high as previous 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 [38].

Cortisol-producing adrenocortical carcinomas, like all adrenocortical carcinomas, have a bad prognosis. Unlike in patients with pituitary Cushing's, 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 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 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 [39].

Less common causes for ACTH-independent Cushing disease, requiring bilateral laparoscopic adrenalectomy, include bilateral macronodular adrenocortical hyperplasia and primary pigmented nodular adrenal disease (PPNAD); the latter 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. MTCs, islet cell carcinomas, and pheochromocytomas can also produce ACTH or 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 [40].

Other rare indications for adrenalectomy include myelolipomas, cysts, and adrenal hemorrhage when the diagnosis is uncertain or pain is an issue.

27.5 Gastroenteropancreatic Tumors

27.5.1 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 [41].

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 [42].

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 [43]. Like with 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 [44].

Duodenal carcinoids are frequently gastrin producing and will be discussed under the heading of Zollinger-Ellison syndrome (ZES). 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 [45].

Gastric carcinoids are most often a consequence of achlorhydria (chronic atrophic gastritis) and secondary hypergastrinemia resulting in 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 [46].

27.5.2 Zollinger-Ellison Syndrome

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; distal pancreatectomy for body and tail masses. Although recurrence rates as high as 50% have been reported, long-term survival is the rule [47].

In MEN I 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. Since recent reports have documented that the most common cause-specific mortality in MEN I patients are the pancreatic and duodenal tumors, many surgeons have promoted early surgical intervention. The Ann Arbor procedure, popularized by Thompson et al. [48], 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. 27.5).

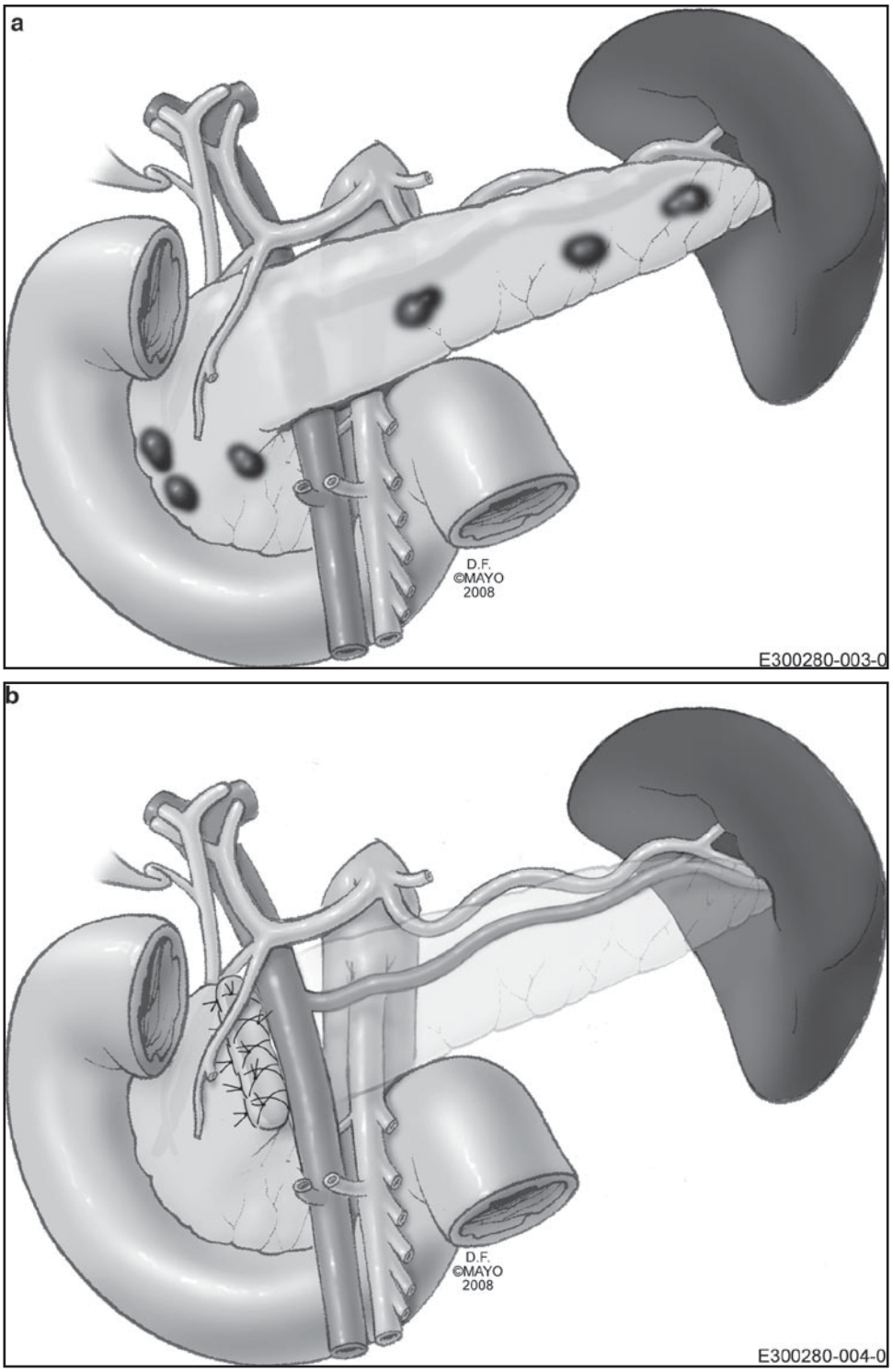


Fig. 27.5 (**a**, **b**) Pancreaticoduodenal procedure for the MEN I pancreas (courtesy of Dr. Norman Thompson, Ann Arbor, MI)

27.5.3 Insulinomas

Insulinomas occur with a frequency of 4 per 1 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,

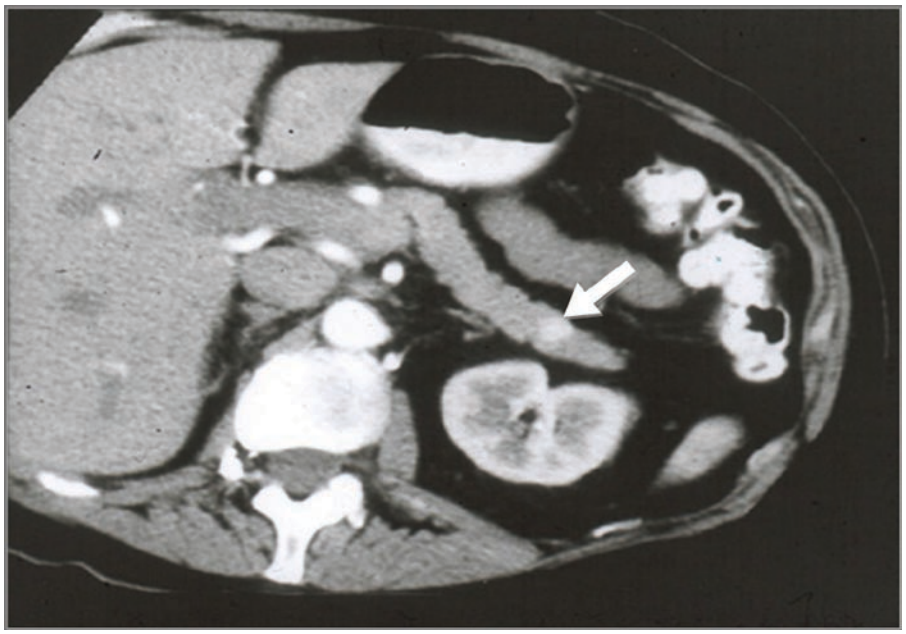


Fig. 27.6 Spiral CT scan demonstrating an insulinoma in the pancreatic tail (courtesy of C.C. Reading, Rochester, Minnesota)

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. 27.6). 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 [49]. 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 [50,51].

MEN I 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.

27.5.4 Glucagonomas

The glucagonoma syndrome is characterized by diabetes, necrolytic migratory erythema, a large malignant alpha-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 (Fig. 27.7a and b) [52].

27.5.5 VIPoma

VIPomas are usually large, malignant, islet cell tumors of the pancreas associated with hypersecretion of vasoactive intestinal polypeptide. 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 [53].

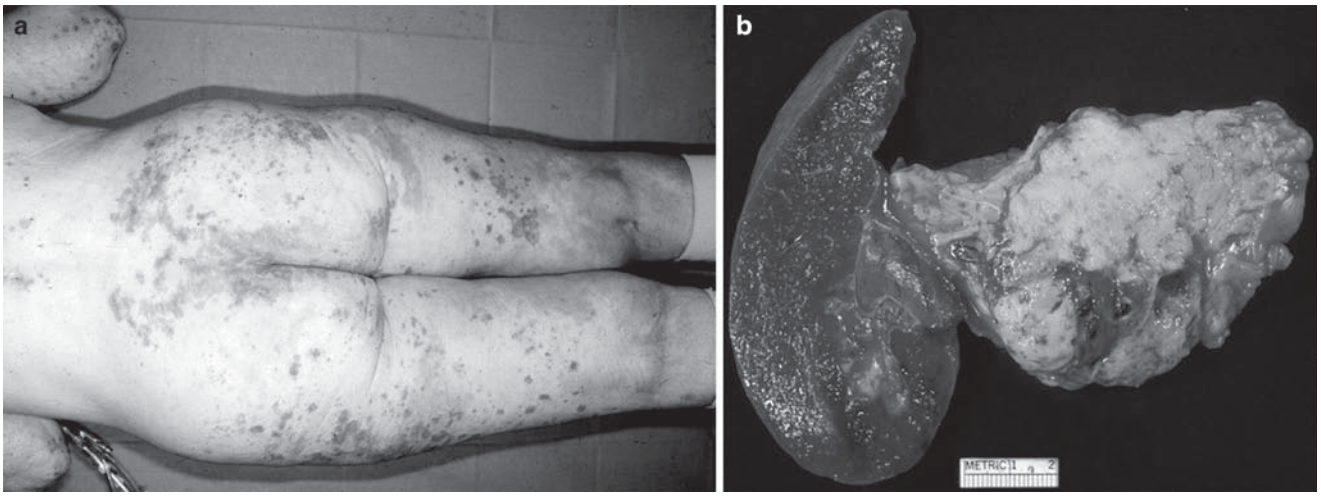


Fig. 27.7 (**a**) Necrolytic migratory erythema. (**b**) Distal pancreatectomy specimen from the patient above with a malignant glucagonoma (rash resolved completely after resection)

27.5.6 Somatostatinomas

These are often large neuroendocrine tumors involving the pancreatic head or duodenum. They occur sporadically but also in association with type I neurofibromatosis. Somatostatin is a potent inhibitory peptide, and, therefore, the clinical manifestations of diabetes, steatorrhea, gallstones, and achlorhydria. Treatment usually requires pancreatoduodenectomy [54].

27.5.7 Less Common Islet Cell Tumors

These include tumors that secrete ACTH, CRF, MSH, CCK, serotonin, and pancreatic polypeptide. Most are large and malignant, and are, therefore, managed accordingly.

27.5.8 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 [55].

27.5.9 Metastatic Islet Cell Tumors

Many patients with islet cell tumors of the pancreas initially present with metastatic disease. Surgical debulking may offer a survival advantage to this group [56]. Furthermore, symptoms related with the various islet cell tumors may also be attenuated with surgical debulking. Treatment of patients with hepatic metastatic disease involves resection of liver lesions with or without radiofrequency ablation or cryoablation. Many authors recommend hepatic resection if greater than 90% of the tumor can be excised and less than 75% of the liver is involved. Other possible therapies for more diffuse metastatic disease include peptide receptor radionuclide therapy which involves administration of octreotide which has been fused to a beta-emitting radionuclide. This procedure however is fraught with morbidities including nausea, vomiting, nephrotoxicity, and lymphocytopenia in some patients. Other possible therapies include hepatic artery embolization and chemotherapy. However, results to date have been disappointing. Finally, patients who are not candidates for curative or cytoreductive surgery or those who are unresponsive to medical therapy may undergo liver transplantation. Metastases of neuroendocrine tumors can be limited to the liver for long durations, thereby opening a window for liver transplantation as a possible therapy. Current data suggest that graft survival at 5 years as well as overall survival in this patient population is not substantially different from transplantation for benign disease in selected patients [57].

27.6 Pituitary

27.6.1 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.

27.6.2 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 [58].

27.6.3 Cushing Disease

ACTH-dependent Cushing's 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 approximately 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. 27.8) [59]. Transsphenoidal surgery is associated with 65–85% long-term cure rates in patients with Cushing's disease [60].

27.6.4 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 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 [61].

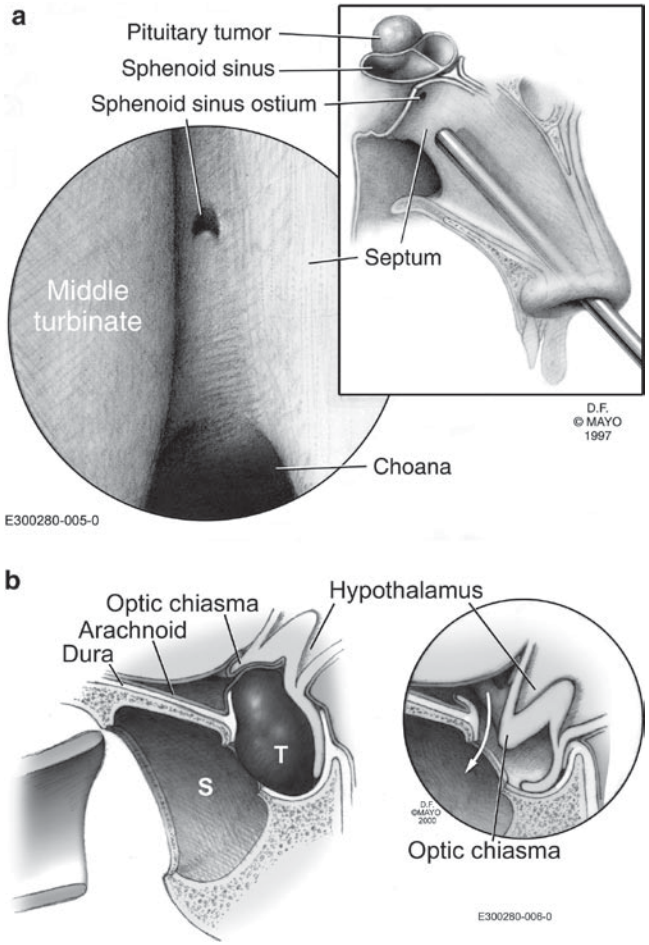


Fig. 27.8 Endoscopic transsphenoidal pituitary surgery (courtesy of Dr. John Atkinson, Rochester, MN)

27.7 Surgery for Diabetes

The first transplantation of pancreatic tissue to a diabetic patient was performed over 100 years ago [62]. Today islet transplantation has evolved considerably and involves the isolation of islets of Langerhan from the pancreas of cadaver donors and transplanting them into recipient livers. The results of these procedures have led to the long-sought-after moment-to-moment control of blood glucose levels, which is more effective than injected insulin. However, these advancements in islet transplantation have led to actualized benefits to less than 0.5% of type 1 diabetics [63]. Limitations to more widespread success of this procedure are primarily associated with lifelong immunosuppressive therapy and inadequate sources or transplantable islets. In addition, long-term benefits have not been realized in these patients with 1-year insulin-free rates ranging from 55 to 100% and 5-year insulin-free rates of only about 10% [64].

To date, the only surgical mainstay for the treatment of diabetes is whole organ transplantation of the pancreas.

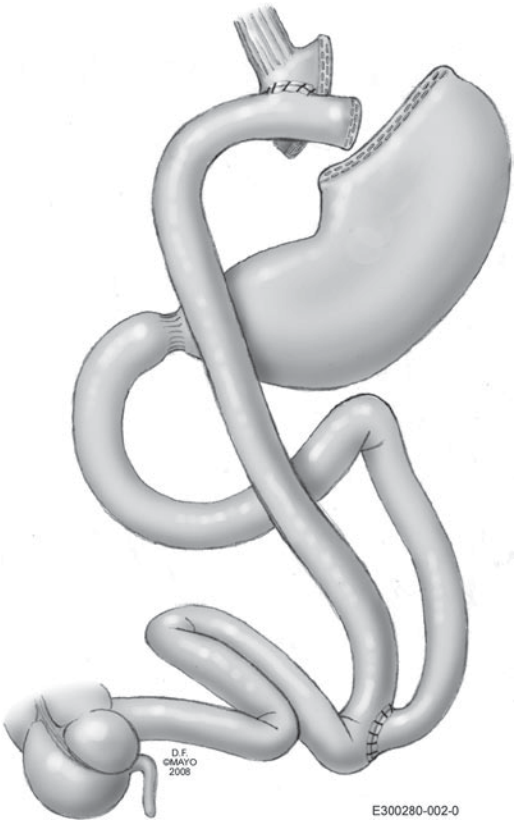


Fig. 27.9 Roux-Y gastric bypass procedure for medically complicated obesity

Whole organ transplantation, particularly when combined with kidney transplants in type I diabetics, has been met with increasing success. The first pancreas transplant was performed in 1966 at the University of Minnesota. Initial results involving pancreas transplantation were poor; however, long-term graft survival has now approached that of other abdominal organ transplants and represents a standard viable surgical alternative for beta cell replacement in brittle diabetics.

Additionally, patients with medically complicated morbid obesity and type II diabetes are achieving dramatic results and cures with combined restrictive and malabsorptive procedures (Roux-Y gastric bypass Fig. 27.9) that facilitate and maintain significant weight loss (>50% of excess body weight) [65].

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Chapter 28
Medical Treatment of Neuroendocrine Tumors

Timothy J. Hobday

28.1 Introduction

The gastrointestinal neuroendocrine tumors (carcinoid tumors and islet cell carcinoma (ICC)) 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 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.

28.1.1 Clinical Considerations

28.1.1.1 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 by resection if the primary tumor is <2 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 carcinoid tumors.

28.1.1.2 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 with induction of anesthesia, after surgery, or hepatic artery embolization, and rarely, spontaneously. Carcinoid crisis results in extreme hemodynamic instability with severe hypertension or hypotension. Prompt therapy with somatostatin analogs can be life-saving [2].

28.1.1.3 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

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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.

28.1.1.4 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].

28.1.1.5 Islet Cell Carcinoma

Advanced islet cell carcinomas can present either through local symptoms (such as obstructive jaundice, pain, or steatorrhea), 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 I (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 llq13 can be offered.

28.1.2 Therapeutic Options for Metastatic Neuroendocrine Tumors

28.1.2.1 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. For patients with the carcinoid syndrome, treatment success (defined by symptomatic control of the syndrome without the need for other supportive measures) occurred in 65% of patients in a clinical trial demonstrating the therapeutic equivalence of the short- and long-acting release (LAR) 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 months of therapy, 12 patients had disease stability after 1 year, and nine patients after 2 years. No tumor regression was seen [5]. It appears that carcinoid tumors may be more likely to demonstrate stabilization of growth and rare regressions than other neuroendocrine tumors. However, a 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% [6].

In a recent small phase III randomized, placebo-controlled trial of Octreotide LAR vs. placebo for minimally symptomatic metastatic carcinoid tumors, a statistically significant improvement in time to disease progression (TTP; 14.3 months vs. 6.0 months) was noted for octreotide LAR therapy [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.

More recently, radiolabeled somatostatin analog therapy has been widely used throughout Europe, though unavailable in the U.S. In a single-institution case series, the use of Lu-177-labeled octreotate in patients with expression of somatostatin receptors on radionuclide imaging resulted in a partial response rate of 30%. Acute toxicity is mild, and long-term follow-up of toxicity is awaited, though rare renal failure, myelodysplasia, and hepatic failure have been reported [8].

28.1.3 Regional Therapies

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 [9]. 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.

28.1.4 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 [10]. 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 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-week 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 that 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 months [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 months vs. 20 months in carcinoid patients and 4 months vs. 20 months in islet cell carcinoma) and overall survival (27 months vs. 49 months for carcinoid patients and 9 months vs. 35 months 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 as currently performed [11].

The addition of hepatic artery infusion of chemotherapy or encapsulated particles containing chemotherapy has been advocated in an attempt to deliver high concentrations of antitumor agents to areas of hypoxic tumor. Several different agents such as 5-fluorouracil (5-FU), streptozocin (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 [12–15]. Direct comparisons of CE and HAE in terms of toxicity and effectiveness are not possible with existing data.

28.1.5 Chemotherapy

28.1.5.1 Carcinoid Tumors

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 nitrosourea 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 [16].

28.1.5.2 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 around 2 years [17]. 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 year, 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 [18]. A study by the Southwest Oncology Group of a combination of STZ/5-FU/ADR also documented a response rate of 31%, but a median survival of only 10.8 months [19]. More recent empiric trials of newer combinations (5-FU/ADR/CDDP) [20] and single agents such as carboplatin [21] and taxol [22] 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.

28.1.5.3 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 [23]. 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 months vs. 6.9 months for 5-FU/STZ, $p=0.001$), and median survival (2.2 year vs. 1.4 year, $p=0.004$) [24]. This remains a standard regimen for islet cell carcinoma.

28.1.5.4 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 months and a median survival of 19 months. These appear to be superior outcomes compared with that expected for these more aggressive histologies [25]. Fjallskog et al. 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 [26]. Toxicity, however, can be considerable with this regimen.

28.1.6 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 III patients with metastatic carcinoid tumors demonstrated biochemical responses, and 15% had objective tumor regression with interferon-α [27]. The same group reported a 47% biochemical response and 12% objective response in other neuroendocrine tumors [28]. 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 [29,30]. Some authors have found the duration of benefit to be disappointingly short and document excessive toxicity with high-dose regimens [31]. More recent investigations

have focused on lower doses of interferon-a 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 a randomized trial of the somatostatin analog lanreotide vs. interferon-α vs. the combination, there was no evidence of additive activity for interferon or the combination vs. lanreotide alone, and toxicity was increased [32].

28.1.7 Future Directions: Targeted Therapy

Clearly, more effective and less toxic therapy is needed for the treatment of metastatic neuroendocrine tumors. We have investigated the pattern of molecular marker expression on a number of resected liver metastases from islet cell carcinomas and carcinoid tumors in an attempt to identify useful hypotheses for study of targeted therapy with novel agents. Frequent expression of vascular endothelial growth factor (VEGF) and its receptors, as well as expression of the epidermal growth factor receptor (EGFR) were noted, and clinical trials were designed with small molecule inhibitors of these targets [33]. A phase II study of gefitinib, a small molecule tyrosine kinase inhibitor (TKI) of the EGFR, in patients with disease progression at study entry, led to disease stability at 6 months in 51% of carcinoid tumors and 28% of ICC. There were few objective tumor responses [34]. The use of sorafenib and sunitinib, which both have TKI activity at multiple receptors, including VEGF-receptors, led to response rates of 2–7% in carcinoid and 11–17% in ICC [35,36]. Another pathway of interest is the mammalian target of rapamycin (mTOR) pathway. A large phase II trial of an oral inhibitor of mTOR in patients with progressive ICC after chemotherapy was performed. A response rate of 8% was seen with single agent everolimus [37].

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. Better understanding of the biology of these cancers is needed to develop better therapy. Trials evaluating combinations of new therapies and carefully comparing standard vs. new therapies are ongoing.

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Chapter 29
Radiation Treatment of Endocrine Tumors

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Malignant tumors of the endocrine glands account for only 2.7% of the estimated 1.44 million new cases of non-skin cancer and 0.1% of the estimated 565,650 cancer deaths in the United States in 2008 [1]. Thyroid cancers account for the vast majority (approximately 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 (RT) in the treatment of some of the more common endocrine tumors.

29.1 Pituitary Adenoma

Pituitary adenomas have an incidence of about 1–14.7 per 100,000 people and an autopsy prevalence of about 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 years 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), adrenocorticotrophic hormone (ACTH) or thyroid stimulating hormone (TSH) resulting in amenorrhea–galactorrhea syndrome, acromegaly, Cushing's disease or secondary hyperthyroidism, respectively. Neurologic sequelae arise from the growth of a mass beyond the confines of the sella, resulting in headache, visual loss (most frequently a superior temporal quadrantanopia progressing to a bitemporal hemianopia with diminished visual acuity), altered hypothalamic function (sleep, eating habits and behavior) due to encroachment on 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 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 an endocrinologic diagnosis is achieved via measurement of basal and provoked hormonal levels; the usual basal hormones checked being prolactin, GH, ACTH, cortisol, TSH and luteinizing hormone (LH)/follicle stimulating hormone (FSH). Confirmation of an 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 para-sellar 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 I 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

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functional outcome assessment). Microadenomas (less than 10 mm in diameter) are more common than macroadenomas (greater than 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 the tumor. The options include pharmacologic inhibition of pituitary hormone secretion, microsurgical excision of the tumor and conformal irradiation. Pharmacologic inhibitors include bromocriptine, a dopamine analog 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 trans-septal trans-sphenoidal approach to resect the entire tumor. Conformal irradiation may be administered either as fractionated external beam RT, hypofractionated stereotactic RT or radiosurgery (linear accelerator photons, Gamma Knife gamma-rays or charged particle based therapy).

A representative fractionated external beam RT plan is outlined in Fig. 29.1. Multiple non-coplanar beams are used to conform the radiation dose three-dimensionally to a treatment volume encompassing the tumor (defined by fusion of the treatment planning CT to a diagnostic MRI) and a margin (to account for uncertainty of tumor extension, patient movement and set-up variability and dose build-up 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 gradients to small volumes so as to protect surrounding normal tissue. A representative Gamma Knife radiosurgery treatment plan is outlined in Fig. 29.2. 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. A dose of 11–20 Gy (hormonally inactive tumors) or 14–30 Gy (hormonally active tumors) is administered in a single treatment. The dose is dependent upon tumor

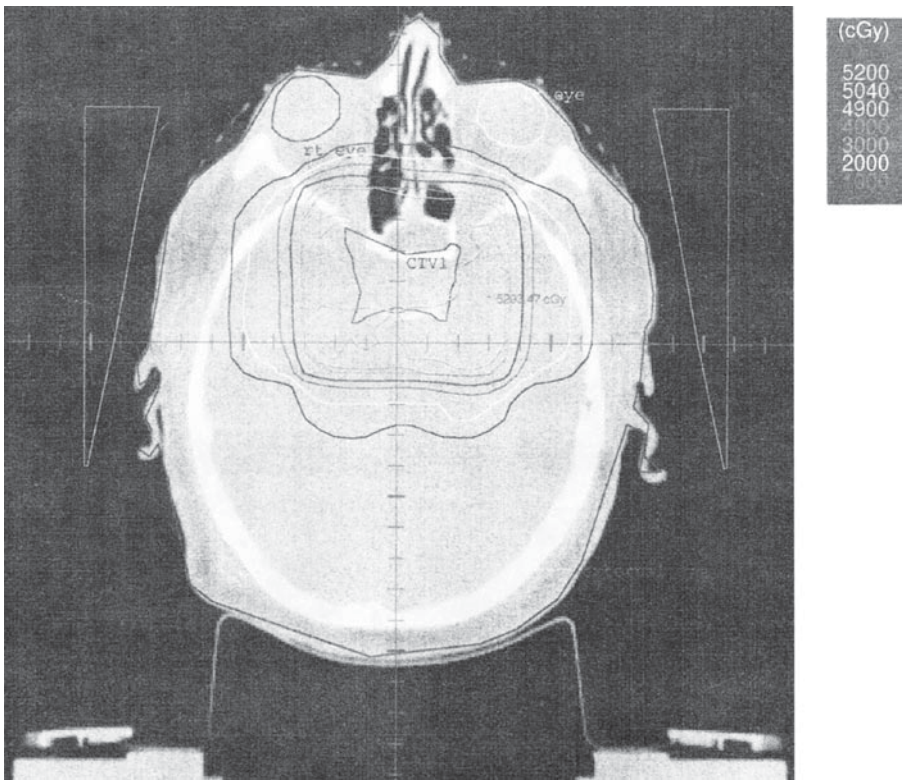

Fig. 29.1 Axial CT scan image obtained for computerized treatment planning for a large non-functioning pituitary adenoma involving the right cavernous sinus. The CTV1 (clinical target volume 1) was obtained by fusion of the treatment planning CT scan images with MRI images obtained in the same patient position (with 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 fall-off beyond the 30 Gy isodose line (*red line*) limits normal brain dose

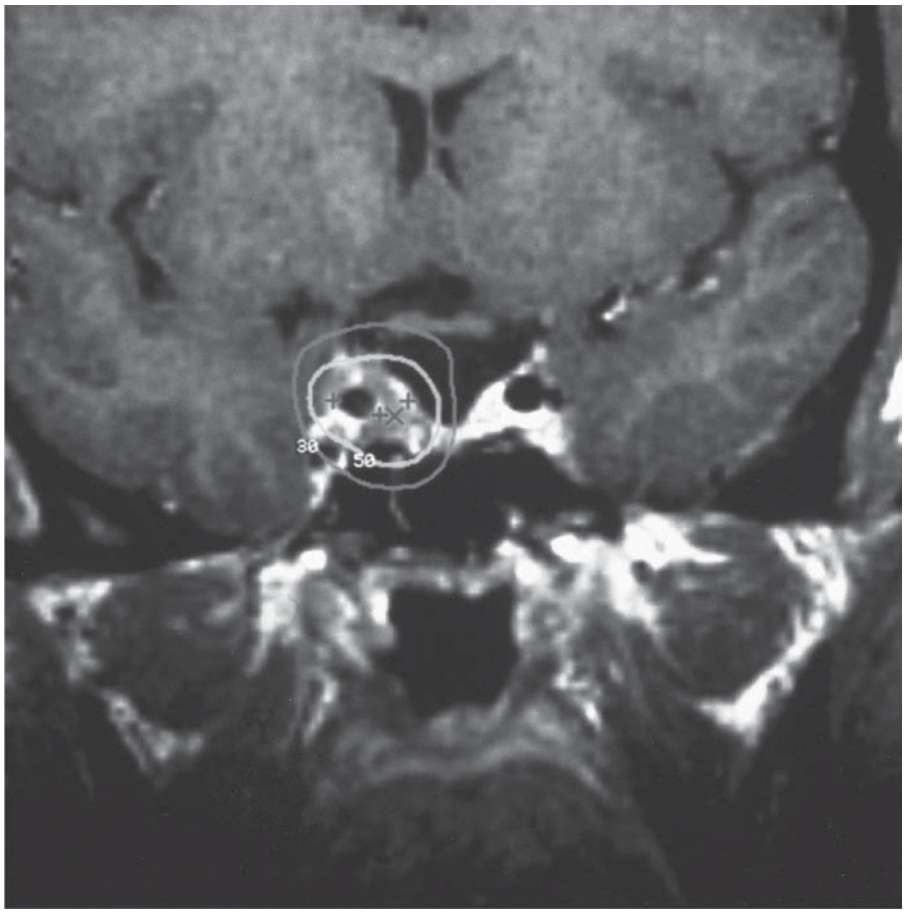

Fig. 29.2 Coronal MRI obtained for computerized treatment planning for a recurrent pituitary adenoma involving the right cavernous sinus. A dose of 15 Gy was administered to the 50% isodose line (*yellow*). The maximum dose to the tumor was 30 Gy. The dose to the optic chiasm was less than 10 Gy (30% isodose line, *green*)

volume, location, extension, prior treatment and histology. Similar treatment can be accomplished using a linear accelerator (linac) to rotate around isocenters placed within tumors or using charged particle beams (protons, helium ions) that also have steep dose gradients (Bragg-peak effect).

Non-secreting tumors may be treated surgically, with RT alone or by a combination of surgery and RT. 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 years [6]. These excellent outcomes are maintained with even longer follow-ups in more recent series as well (15-year local tumor control rate of 93% [7]). Total doses range from 45 to 50.4 Gy administered as 1.8 Gy per treatment, five treatments per week over 5–6 weeks.

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 [8]. Macroprolactinomas with high pre-treatment prolactin levels and mass effect or apoplexy are treated surgically and may be given bromocriptine preoperatively to decrease prolactin levels and tumor size. Despite this, incomplete surgical resection of macroprolactinomas results in high recurrence rates (averaging roughly 50%) [9]. 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, RT or surgery.

Primary RT is usually reserved for medically uncontrolled patients who are poor surgical candidates, and leads to a 5-year 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 pre-treatment levels of GH, and 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]. These results have been fairly consistent across a variety of RT techniques including stereotactic radiosurgery (Gamma Knife, CyberKnife, and linac-based radiosurgery), fractionated RT and proton therapy [10]. 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 fractionated doses up to 45–50 Gy, beyond which there does not seem to be any increased response [11–15]. 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 nerve or chiasm. Encouraging results have been reported with all forms of radiosurgery for primary treatment. Gamma knife radiosurgery literature suggests roughly 40–100% GH normalization at 2 years [16–18] and 30–60% prolactin normalization at 2 years [17, 19]. These series report local control rates of greater than 90%. Linac-based radiosurgery also results in greater than 80% local control and comparable hormone normalization rates, albeit with greater neurologic toxicity (optic nerve damage and temporal lobe necrosis) when single isocentric techniques are used [20, 21]. Although lower complication rates were noted in one series [22], there has been a shift towards the use of fractionated stereotactic radiotherapy or multiple isocentric techniques [20, 23]. Reported hormone normalization rates at 5 years are 50% for acromegaly and 80% for Cushing's disease with proton beam therapy [24]. While these techniques have traditionally been reserved for retreatment of previously irradiated cases or instances where recurrence is noted in the cavernous sinus, there is insufficient durable control data to make them obvious choices as first line RT [25].

Using modern equipment and techniques, the toxicity of RT is expected to be rare. Fortunately, most patients with pituitary adenomas can expect to be cured of their neoplasm. Consequently, understanding the adverse effects of treatment becomes an increasingly relevant consideration while choosing appropriate management strategies. Hypopituitarism is noted in 13–56% of patients and may be the result of a combination of factors including pretreatment tumor effect, surgery and RT [6]. Furthermore, the risk of post-RT hypopituitarism is dose-, fractionation-, radiation quality- (charged particle vs. photon) and time-dependent. Anterior pituitary hormone deficiency is the most common manifestation of RT-induced hypopituitarism. Deficiency of GH, the most vulnerable anterior pituitary hormone, is seen at doses as low as 30 Gy at the hypothalamic–pituitary axis and particularly in children, virtually all of whom (80–100% vs. 32% in adults) develop hypopituitarism [26]. TSH and ACTH deficiencies occur after more intensive RT (>60 Gy), manifesting in 30–60% of patients on 10-year follow-up [26]. Gonadotropin deficiency is noted at doses greater than 30–50 Gy. Interestingly, doses less than 30 Gy can induce precocious puberty in prepubertal girls [26]. In a recent series reporting a median follow-up of 10.5 years, 97% of patients with pituitary adenomas treated postoperatively with RT developed hypopituitarism (partial hypopituitarism in 36% and panhypopituitarism in 61%) with somatotropic function being most commonly affected while gonadal axis is disturbed the earliest [7]. These effects are noted even when the irradiated pituitary gland does not harbor an adenoma but is merely within the treatment field (surgical and RT). In fact in a contemporary series of patients with non-pituitary brain tumors treated with RT, the incidence of GH, gonadotropin, ACTH and TSH deficiencies and hyperprolactinemia was 32, 27, 21, 9, and 32%, respectively [27]. With multiple non-coplanar beams the risk of radiation-induced brain necrosis is believed to be less than 1% [28]. Limiting the dose to the optic nerves and chiasm to 50 Gy reduces the risk of optic pathway dysfunction to less than 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 [29, 30]. Data derived from older techniques suggests a cumulative risk of developing a secondary brain tumor over the first 20 years 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 radiation-induced malignancies is about 10 years, it is not surprising that reports of radiosurgery-induced second malignancies are sparse in the literature. Potential advantages of radiosurgery, as compared to conventional RT, include lower doses to the surrounding normal tissues (brain, functioning pituitary, optic apparatus), shorter treatment time (1 day versus several weeks), and more rapid normalization of elevated hormone levels.

29.2 Thyroid Cancers

It is estimated that 34,370 new cases of thyroid cancer will be diagnosed in the United States in 2008 and 1,590 patients will die of thyroid cancer [1]. Ninety five per cent of all diagnosed cases are differentiated (follicular or papillary) cancers. 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 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 Chap. 23 for details). The most common histologies are papillary, follicular, medullary and anaplastic carcinomas, and lymphomas (diffuse large cell non-Hodgkin's type and mucosal-associated lymphoid tissue (MALT) lymphomas).

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 [31]. Other stratification strategies include AGES (age, grade, extent, size) [32], AMES (age, metastases, extent, size) [33] and MACIS (metastases, age, completeness of resection, invasion, size) [34], which have all 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 [35].

The initial treatment of choice is surgery, which usually involves a total or near-total thyroidectomy (including a lymph node dissection for papillary carcinomas that have a predilection for lymphatic spread) [36]. It is customary to obtain a whole body radioiodine scan (WBS) a few weeks after surgery to quantify residual thyroid tissue and detect 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 I^{131} scanning by eliminating uptake by the 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 [35, 37–39], lower pulmonary metastases rates [37, 40] and decrease mortality in high risk patients [37, 39]. The treatment usually involves use of 30–100 mCi of I^{131} depending on the size of the remnant. To enhance therapeutic efficacy, it is preferred that low dose (2–3 mCi) I^{131} is used for the WBS and minimal iodinated contrast is used for staging CT scans. A 4–7 day 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. Follow-up includes clinical examination aided by an ultrasound if necessary, thyroglobulin level measurement, anti-thyroglobulin antibody level measurement, TSH check and WBS after thyroxine withdrawal or with recombinant human TSH administration.

While 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 RT up-front in the management of this subset of patients is to:

- Treat tissues that are known to take up less radioiodine (areas of extrathyroidal or extranodal extension, which are known poor prognostic indicators [33, 41–45]),
- Treat carcinomas that fail to concentrate and retain radioiodine (20% of all differentiated thyroid cancers, patients with Hurthle cell tumors and patients above the age of 40 who commonly have less sodium iodide symporter [46–48]),
- 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 [49]), and
- Spare some of the morbidity associated with uncontrolled local-regional cancer, such as obstruction of the esophagus and/or trachea, neurovascular compromise, pain, hemorrhage, need for a laryngectomy, and the need for repeated surgical procedures.

Non-randomized studies have identified a potential role for adjuvant treatment of high-risk patients with external beam RT, documenting improved local control with no survival benefit [6, 50–58]. In one study, there was a cause-specific survival benefit noted among papillary cancers with microscopic residual cancer [38]. The inclusion criteria for receiving external beam RT have included advanced age (>40 years), extrathyroidal extension, and a high score on a scoring system for prognostic variables.

Local recurrence has purportedly declined from 20–51% to 4–8% with the addition of RT in these studies, translating to a 65–85% decrease in local recurrence.[38, 50–64] 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. A prospective, randomized, clinical trial may be needed to assess the benefit of such a treatment approach. However, large

randomized studies of RT vs. no RT are difficult to perform. In the absence of prospective data, we recommend treating high-risk locally advanced thyroid cancer patients with adjuvant external beam RT since the incidence of locoregional failure remains high despite aggressive surgical management and radioiodine ablation, and modern RT techniques permit treatment with acceptable toxicity profiles. Since the incidence of distant recurrences also remains high among these patients, there may also be a role for a combination of RT with systemic therapy such as the multikinase inhibitors that have demonstrated promising clinical activity [65]. In the future, we will need to discover molecular markers to identify and predict aggressive tumor behavior similar to the recent identification of gene expression profiles to differentiate between benign and malignant thyroid tumors [66].

In addition to the adjuvant treatment mentioned above, external beam RT is employed in the treatment of advanced inoperable primary or recurrent local-regional disease [38, 52, 53, 67], radioiodine-resistant progressive disease [68] and bone, lung, nodal or brain metastases. Treatment of the thyroid gland and its draining lymph node areas has historically been a challenging undertaking, inspiring a number of novel strategies to achieve adequate tumor dose while minimizing the dose to normal tissues. Opposed anterior-posterior fields with lead blocks anteriorly for the larynx, anteriorly and posteriorly for the lungs and a posterior block for the spinal cord, lateral fields, anterior oblique fields and centrally shielded arcs with one or two isocenters 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, including CT-guided IMRT and Intensity-Modulated Arc Therapy) treatments. The last of these, intensity-modulated RT, is a method where normal tissue dose constraints and tumor target dose prescription goals are utilized by a forward planning computer to develop an ideal treatment plan, instead of manually and empirically selecting potential treatment beams and hoping to limit normal tissue doses [69, 70]. A representative 3-D conformal treatment plan is shown in Fig. 29.3. Typical total doses are 50–60 Gy administered as 2 Gy per treatment, 5 days per week, over 5–6 weeks for microscopic disease (adjuvant treatment). Total doses in the range of 60–70 Gy over 6–7 weeks are employed for gross residual disease, with roughly 60% relapse-free survival at 5 years and 40% at 15 years. Treatment volumes typically encompass all lymph node regions "at risk" (typically from the hyoid bone to the carina, levels II, III, IV, V, and VI). While there is no definitive evidence of a dose–response relationship between radiation dose and local control probability, there is some suggestion that higher doses are associated with lower local recurrence rates [53, 58].

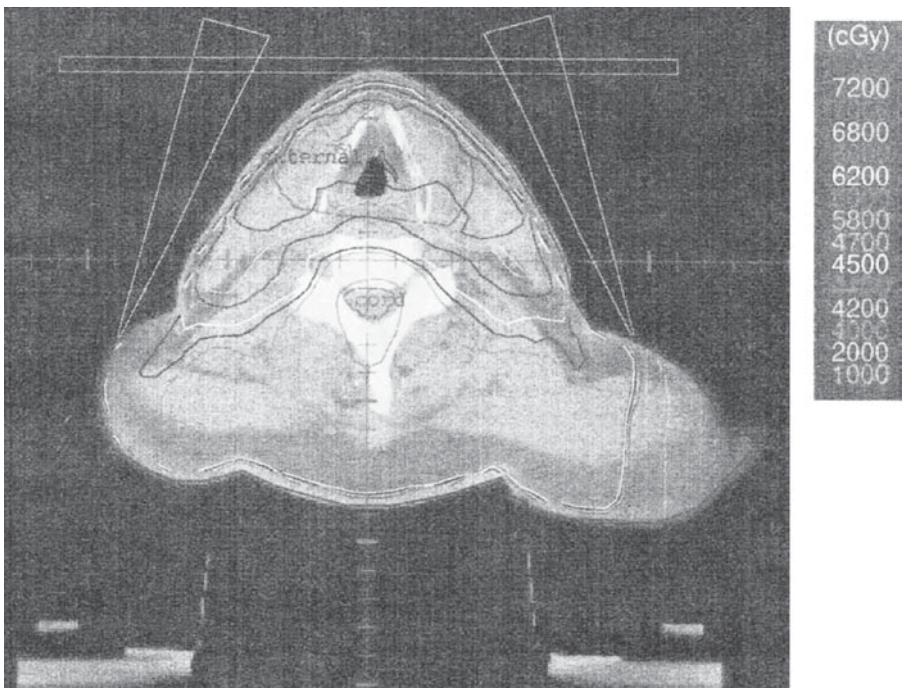

Fig. 29.3 Axial CT scan image through the thyroid gland in a patient with thyroid bed recurrence after thyroidectomy for a papillary thyroid cancer. A dose of 70 Gy was administered to the thyroid recurrence encompassed by the innermost *dark purple line*. A dose of 66 Gy was administered to the thyroid 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 less than 47 Gy (*green line* around the cord)

With the increasing availability of IMRT and proton radiotherapy, dose escalation may be more readily achievable in the adjuvant setting [58, 70, 71]. However, ideal planning target volumes and doses remain inadequately characterized. Early experiences with this approach seem to suggest that escalation of dose to subclinical sites of possible involvement (nodal regions outside the thyroid bed to 54 Gy), the margin-negative thyroid bed (60–63 Gy), margin-positive regions (66 Gy) and gross disease (70 Gy) is technically feasible [70]. One treatment is given each day, 5 days a week, 1.8–2.0 Gy per treatment. The authors maintained a low threshold for placement of tracheostomy or percutaneous endoscopic gastrostomy tubes and recommended vigilant proactive clinical care to minimize and ameliorate treatment-related toxicity. 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 [72]. Acute RT reactions include dermatitis, laryngitis and esophagitis. Late sequelae are uncommon but may include laryngeal edema, cartilage necrosis, esophageal stenosis, myelitis, and pulmonary fibrosis.

We have a limited experience using RT in the management of Hurthle cell carcinoma of the thyroid gland. We have found it to be a radiosensitive malignancy. We have treated five patients with adjuvant RT. 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 months following RT. We have treated 5 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 months). We have treated 14 patients with 95 palliative courses of RT for symptomatic metastases. All patients experienced relief from pain and other symptoms for a median of 12 months (range, 0.5–68 months). Overall, 33% of the sites treated required retreatment. If a dose of more than 25 Gy is utilized, such as a total dose of 30 Gy given as ten 3 Gy treatments over 2 weeks, only 16% of metastatic sites will require retreatment and the median duration of palliation is extended to 14 months [73]. A more recent report on a large series of patients with Hurthle cell carcinomas treated with multiple modalities failed to identify a survival benefit with the use of external beam RT but its role in improving local control was not reported [74]. It was, however, noted that patients receiving adjuvant radioactive iodine ablation had better survival rates than those who did not receive it. In the absence of prospective data, the authors recommend treating patients with postoperative thyroid bed uptake with adjuvant radioiodine ablation.

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 which produce calcitonin, accounts for approximately 10% of all thyroid malignancies. An estimated 75% of these are sporadic while the remaining 25% are familial and tend to be multifocal bilateral tumors. The hereditary medullary thyroid cancer syndromes include MEN type 2A, MEN type 2B and familial medullary thyroid cancer without other malignancies. MEN type 2A patients tend to have accompanying pheochromocytomas and parathyroid hyperplasia/adenomas. MEN type 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 pre-operative measurements of serum calcitonin and carcino-embryonic antigen (CEA) levels; screening for pheochromocytomas with urinary catecholamine measurements and screening for MEN2 syndromes by genetic testing for *ret* proto-oncogene mutations. Primary treatment involves total thyroidectomy with parathyroid autografting preferably before the age of 5 years, central lymphadenectomy and in larger tumors with nodal involvement, 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 which 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 overall survival in one series [75]. 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% [76]. However, 30% local recurrence rates within an irradiated field after 54 Gy [77] and even a decrease in survival among patients treated with RT [78] 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 [79, 80]. While adjuvant treatment of microscopic residual disease with RT is not routine [81], some suggest that this may be a reasonable option for patients at high risk of local-regional relapse [76, 82]. Radioimmunotherapy with I^{131}-labeled CEA monoclonal antibody protocols are an option in case of nonoperative widely metastatic medullary thyroid cancer and somatostatin analogs are used for symptomatic disease [79].

Anaplastic thyroid cancer has a universally dismal prognosis with survival beyond 1 year being very uncommon. The hallmark of the disease is a rapidly enlarging mass with pain and pressure effects on the upper aerodigestive tract. While there is no optimal treatment available, a multimodality approach consisting of surgery, RT and chemotherapy

seems to be the preferred approach. While most series report long-term survivors only among surgically resected patients [83–88], the high morbidity of this procedure in the face of a poor prognosis may warrant its judicious use [89]. Even in the presence of distant disease, since most patients die from uncontrolled local symptoms, achieving local control is not only an important objective of treatment but may also contribute to short-term survival [90]. RT in conjunction with doxorubicin chemotherapy results in improved local-regional control and survival in some series [80, 88, 89] though not in others [91]. Hyperfractionated RT (more than one treatment per day at a reduced dose per treatment) has been employed in some series as a means of keeping up with the high proliferation rate of these aggressive tumors, while limiting normal tissue toxicity [44, 83, 84, 92]. In the most recent update of this approach, outcomes were reported in three groups of patients based on the RT fraction size and era of treatment – group A (1.0 Gy per treatment, 1984–88), group B (1.3 Gy per treatment, 1988–92), and group C (1.6 Gy per treatment, 1992–99) [93]. Surgery was performed wherever possible and patients in groups A and B received 30 Gy preoperatively and 16 Gy postoperatively while patients in group C received 46 Gy preoperatively. Local control was achieved in five of 16 patients (group A), 11 of 17 patients (group B), and 17 of 22 patients (group C). In patients who underwent resections, local control was achieved in five of 9 patients (group A), 11 of 14 patients (group B), and 17 of 17 patients (group C). In the context of aggressive multidisciplinary management of anaplastic thyroid cancer, the preoperative use of accelerated (more than one treatment per day at normal or near normal doses per treatment to decrease overall treatment time) and hyperfractionated treatment regimens and technological improvements in RT delivery could contribute to improved treatment outcomes. Despite the promise of improved outcomes with aggressive multimodality treatment regimens, the outlook for anaplastic thyroid cancer patients is still very grim. This underscores the need for continued exploration of novel therapeutic strategies.

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 mucosal-associated lymphoid tissue (MALT) lymphomas or diffuse large cell lymphomas of intermediate or high-grade morphology. Though most of these tend to be limited to the thyroid (Ann Arbor stage IE), a complete work-up 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 non-surgical.

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 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 RT to a localized area including the thyroid and regional nodes. Combined modality therapy improved disease-free survival and time to progression without improving overall survival [94]. The SWOG study compared eight cycles of CHOP to three cycles of CHOP followed by 40–55 Gy localized RT [95]. While there was an improved failure-free survival and overall survival rate noted at 4 years for combined modality therapy, this advantage was lost with further follow-up extending up to 10 years. 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 years and the lower toxicity noted in this study [96] as well as the continued use of this strategy as a platform upon which other therapies are added to improve treatment outcomes further [97]. The standard treatment for bulky stage II or stage III/IV diffuse large cell lymphoma is eight cycles of CHOP chemotherapy [98]. CHOP chemotherapy plus rituximab (a chimeric anti-CD20 IgG1 monoclonal antibody) has been shown to improve event-free survival and overall survival in elderly patients [99].

The treatment options for stage I/ II low-grade MALT lymphomas include localized low-dose RT (25–35 Gy), oral chlorambucil or intravenous chemotherapy (cyclophosphamide, vincristine and prednisone) [100, 101]. Given the ease of administering low-dose RT, 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 [102].

29.3 Parathyroid Cancers

Carcinoma of the parathyroid gland is a rare cause of hyperparathyroidism, accounting for less than 1% of patients with hyperparathyroidism. Patients with parathyroid carcinoma usually present with profound symptoms of hyperparathyroidism with highly elevated serum 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. Due to the rarity of the disease, the technically challenging nature of the surgical procedure and the occasional late intraoperative recognition of the 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 [103]. 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 RT should be considered [103]. Many studies addressing the role of RT in the management of parathyroid cancer have reported minimal success at reducing hormone production and tumor growth [104–110]. However two studies and our own experience suggest that adjuvant RT to the tumor bed might decrease the strong predilection for local progression and death due to hypercalcemia [111–113]. Indications for postoperative RT 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 (levels II through VI) 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.

29.4 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 analogue octreotide and chemotherapy (interferon-alpha, streptozotocin, 5-fluorouracil) may help control hormone secretion and stabilize tumor growth. There is a scarcity of literature on the use of RT in this setting, with some case reports documenting good outcomes [114, 115]. As a standard practice, RT is not considered an integral part of treatment of pancreatic islet cell tumors.

29.5 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 foregut carcinoid tumors (originating in the lungs, bronchi, thymus, stomach or duodenum), midgut carcinoid tumors (originating in the small intestine, appendix, and proximal colon) and hindgut carcinoid tumors (originating in the 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-hydroxy indoleacetic 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 analogue treatment and in situ RT. In situ RT capitalizes on the expression of the somatostatin receptor type 2 on greater than 80% of all carcinoid tumors, allowing for its targeting with its ligand bound to a radioisotope emitting short-range decay particles to tumor cell nuclei. In^{111}-pentetreotide and a Y^{90}-DOTA-lanreotide derivative are two such radiolabeled somatostatin analogues shown to have significant early response rates (objective and clinical symptom benefit) with minimal toxicity [116–118]. I^{131}-meta-iodobenzylguanidine (MIBG) (used routinely for diagnostic imaging purposes) has also been used as a means of administering therapeutic-range doses of in situ RT to tumors that exhibit high uptake rates [119–121]. 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 [122–124]. External beam RT has generally been reserved for amelioration of symptoms caused by bone, brain and skin metastases.

RT 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-week period of time. Malignant tumors require a total dose of 60–70 Gy administered as 2 Gy per treatment day over a 6–7-week period of time. RT 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. RT can palliate distressing symptoms due to bone or soft tissue metastases for almost all malignant tumors. A typical dose would be 30 Gy given as ten treatments of 3 Gy each over 2 weeks. 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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Chapter 30
Future Directions in Endocrine Pathology

Ricardo V. Lloyd

30.1 Introduction

During the past 5 years, major advances have continued to be made in understanding the pathogenesis of endocrine tumors and in developing methods to treat endocrine 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 and investigations into microRNA functions have provided new insights about the functions of many genes and protein molecules. Advances in tumor localization, developments in pharmacology, and gene therapy approaches have all contributed to new and effective methods of treating endocrine disorders.

30.2 Pathogenesis of Endocrine Tumors

As the chapters in this book have clearly shown, the pathogenesis of endocrine tumors is extremely complex, and the mechanisms of tumor development in most endocrine systems are still being investigated. To make the most rapid progress in elucidating the pathogenesis of endocrine tumors, investigators will have to concentrate on specific organs or tissues as the mechanisms of genetic instability leading to tumorigenesis are quite different in various endocrine tissues. Recent studies indicate 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 and in other endocrine tumors [1–3 and Chap. 5]. For example, the p27 gene plays a significant role in tumor development in various endocrine tissues and its interaction with menin and cell cycle proteins [4–6]. In the case of tumor suppressor genes such as the multiple endocrine neoplasia (MEN) type I, the *MEN-1* gene affects many endocrine tissues including the pituitary, parathyroid, pancreatic islets, and adrenal medulla. Genetic alterations in these *MEN-1* 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 llql3 has supported a role of the *MEN-1* tumor suppressor gene in a subset of sporadic parathyroid tumors [9]. Similar studies in gastrinomas have shown that mutations in the *MEN-1* gene are important in a proportion of sporadic gastrinomas [10]. In contrast, tumors of the pituitary gland in patients with *MEN-1* are associated with alterations in the *MEN-1* gene whereas sporadic pituitary tumors rarely have alterations in the *MEN-1* gene [11,12]. The *MEN* 2A, 2B, and familial medullary thyroid carcinoma gene, which is the RET protooncogene, has been associated mainly with familial disease affecting the thyroid C-cells and adrenal medulla and to a lesser extent with sporadic diseases affecting these tissues [13–15]. Some genes (and proteins) such as *LGALS3* (galectin-3) have been associated with tumor development and/or progression in several endocrine tissues such as pituitary, thyroid, and parathyroid [16–19]. Newer approaches summarized as follows should lead to new aspects of gene discovery and accelerate knowledge about the etiology and treatment of endocrine tumors.

30.3 DNA Microarray Technology

Advances in DNA array technology have allowed systematic approaches to biological discoveries that will have a major impact in biology, pharmacology, and medicine [20–24]. 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

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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 [25–28], pancreatic islets [29], adrenals [30], and thyroid [31–33]. Studies in the pituitary have uncovered genes that are uniquely expressed by different types of pituitary tumors, which reinforce 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 beta (TGF beta), thioredoxin-interacting proteins, and islet amyloid polypeptide. These studies highlight the importance of the TGF beta family in the regulation of islet cell function [29]. Recent DNA array studies with papillary thyroid carcinomas [31–33] 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 [29]. DNA array studies of thyroid tumors may provide data for a more accurate and reproducible classification of these tumors [33].

30.4 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 [33–36]. New technological advances have been made in proteomics of human thyroid tissue which should help to advance the field [33]. The field of proteomics holds great promise in providing new insights into the discovery and analysis of new proteins in endocrine cells and tumors.

30.5 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 [34–38]. 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 [39,40]. 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.

30.6 MicroRNA

MicroRNAs (miRNAs) are noncoding RNAs that are single-stranded 19–26 nucleotides molecules which regulate expression of target genes. They have been identified in the last few years in vertebrates, invertebrates, and plants [41]. MiRNAs regulate the levels of many of their target transcripts as well as the proteins encoded by these transcripts. Some miRNAs participate in basic biological processes such as cell proliferation and apoptosis. Recent studies have shown that they have the potential for cancer detection, diagnosis, and prognosis [41–44].

A great deal of experimental evidence is accumulating showing the importance of microRNAs in regulating tumor development and progression in endocrine tumors [45–50]. In studies of thyroid tumors specific microRNAs such miRNA-22 and -222 have emerged as potential diagnostic and therapeutic targets [45–47]. In pituitary tumors let-7 has been shown to interact with HMGA2 [48] and specific miRNAs including 15 and 16-1 are altered during progression from normal pituitary to pituitary adenomas [48–50].

Other forms of microRNAs such as RNA interference have been used because 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 [51,52]. Mechanisms of silencing include RNA-induced silencing complex (RSC) which converts the silencing trigger to approximately 21–25 nucleotide RNAs, and these small interfering RNAs (siRNAs) operate by joining an effector complex RSC that guides the complex to homologous substrates [51,52]. More knowledge about the function of many genes in endocrine tumors will soon be forthcoming with this new technology.

30.7 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 [53–55]. The use of octreotide to image growth hormone producing pituitary tumors and sestamibi scans to localize parathyroid tumors [56] 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 [57]. Gamma probes have been used to identify normal parathyroid glands during surgery [58]. MicroRNAs have recently been used as imaging agents for thyroid carcinomas [59]. We can anticipate that these techniques will continue to improve and allow us to detect smaller endocrine lesions in the future.

30.8 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 the analysis of traditional drugs have shown that these are more complex than was previously realized. Although octreotide is useful for some pituitary, pancreatic, 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 [60–63]. 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 [64,65].

30.9 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 [66–69]. 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 [66]. 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 [66]. Other investigators have used adenoviral beta-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 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 [67,68].

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 [69]. 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 [69].

30.10 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 I diabetes mellitus [70–74]. 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 [72] and neural stem cells derived from adult mouse brain [73] are promising areas of exploration, especially given the close relationship between the neural and endocrine systems. Adult stem/progenetic cell-like populations have recently been described in human thyroids [74] and in many other endocrine tissues [75].

In summary, the many recent advances elucidating the pathogenesis of endocrine tumors and the development of new technological advances such as high-throughput RNA expression profiling, proteomics, and microRNAs should rapidly

increase our knowledge of endocrine tumor pathogenesis and increase the therapeutic options. 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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