Molecular Aspects of Thyroid Carcinogenesis

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Contents

Abstract

The thyroid is one of the largest endocrine glands in the body and of highest importance to healthy life by regulating energy metabolism, protein synthesis, and hormone sensitivity. Tumors of the thyroid are rare, accounting for only ~2% of all tumors being diagnosed worldwide. Remarkably, this cancer entity is more frequent in women than in men, with incidence ratios of approximately 3:1. This chapter will introduce the main features of thyroid cancer development, especially focusing on altered molecular signaling and epigenetic variations.

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10.1 Anatomy and Physiology of the Thyroid Gland

The thyroid gland is a key organ of the endocrine system and therefore an essential regulator of numerous physiological processes, including energy metabolism, protein synthesis, and hormone sensitivity. The principal hormones produced by the thyroid itself are triiodothyronine (T_3) and thyroxin (T_4), generated from the amino acid tyrosine and elemental iodine attachments [1]. The third major hormone secreted by the thyroid is calcitonin, involved in calcium homeostasis. Calcitonin is produced in the so-called C-cells of the thyroid gland [1]. The organ is built up of two lobes that are positioned on both sides of the trachea, closely underneath the larynx. The lobes are connected by a small band of thyroid tissue called isthmus. Each lobe has a size of 4–6 cm in length; the whole organ has a weight of 15–30 g in adults, but can be vastly increased in conditions of disease [2].

Microscopically, the thyroid tissue is made up of numerous follicles of varying sizes. The follicular lumen containing colloid is framed by one layer of follicular cells. The C-cells are located on top of or in between follicular cells; they are associated with a particular follicle. The name parafollicular cells for C-cells, which can be found frequently in the literature, is thus incorrect. Both normal and hyperplastic C-cells have an intrafollicular localization [3] (Fig. 10.1j).

Within the colloid the thyroid hormones, T_3 and T_4 are stored. These two hormones are the only iodine-containing compounds involved in physiologic processes [4]. Iodine taken up through nutrition is absorbed through the small intestine and shuttled to the thyroid via the bloodstream. The thyroid is very well supplied with blood, so that it only takes approximately one and a half hour for the whole blood to pass through the gland. Within the thyroid, iodine gets stored, oxidized, and finally incorporated in precursors of T_3 and T_4. When stored in colloid, the hormones are bound to thyroglobulin protein Tg; secreted hormones are first separated from Tg through proteolysis, but will interact with other proteins in circulation. In fact, 80% of circulating T_3 and T_4 are associated with thyroxine-binding globulin (TBG); 10% each are coupled to albumin or prealbumin [5].

Hormone release from the thyroid is tightly regulated in a negative feedback loop, called the hypothalamic-pituitary-thyroid axis. As soon as the hypothalamus recognizes low levels of circulating T_3 and especially T_4 hormone, it secretes thyrotropin-releasing hormone (TRH). TRH binding triggers the release of thyroid-stimulating hormone (TSH) in the pituitary. Consequently, TSH acts on the thyroid, which is stimulated to release T_3 and T_4, thereby increasing their concentration in the bloodstream and slowing down this regulatory circuit. The negative feedback acts directly on the hypothalamus but also signals to the pituitary gland [6].

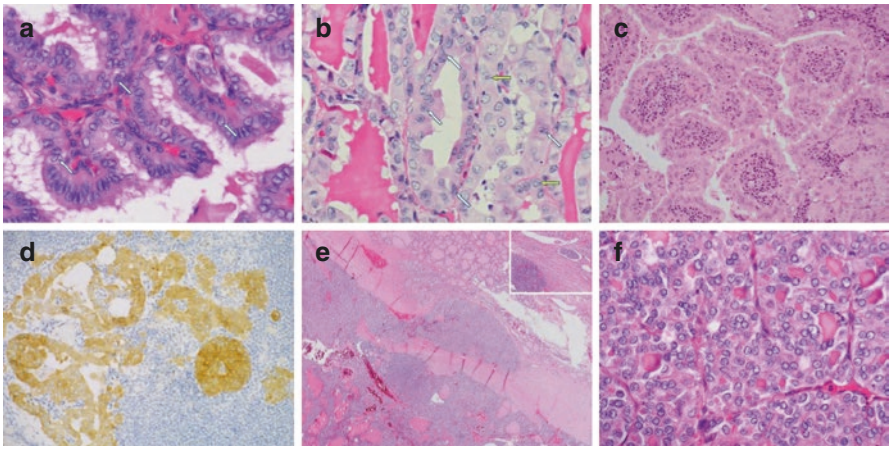

Fig. 10.1 (**a**) Papillary thyroid carcinoma (classic variant). The tumor is composed of papillary structures with gentle fibrovascular stalks. The cell nuclei display a number of characteristic alterations including elongation, overlapping, irregular contours, chromatin clearing, and numerous infoldings of the nuclear envelope, i.e., so-called grooves (*white arrows*). HE stain. (**b**) Papillary thyroid carcinoma, follicular variant. The tumor shows follicular architecture; no papillary structures are seen. However, the tumor cell nuclei display typical alterations of a papillary carcinoma including grooves (*white arrows*) and eosiniphilic intranuclear cytoplasmic inclusions (*yellow arrows*). HE stain. (**c**) Papillary thyroid carcinoma, tall cell variant. The cytoplasm of the tumor cells is larger and more eosinophilic than in conventional type. The height of the cytoplasm is approximately three times as much as its width. (**d**) Lymph node metastasis is typical for papillary carcinoma. The presence of BRAF mutation can be detected by means of immunohistochemistry (positive cytoplasmic +/− nuclear stain with anti-BRAF V600E VE1 monoclonal antibody, Ventana, cat.no. 790-4855). (**e**) Follicular thyroid carcinoma. The tumor usually consists of follicles of variable size. Penetration of the tumor capsule and blood vessel invasion (*inset*) resulting in distant hematogeneous metastasis is typical for this type of tumors. (**f**) Follicular thyroid carcinoma at high-power magnification. The cell nuclei do not display the typical features of a papillary carcinoma. (**g**) Anaplastic (undifferentiated) thyroid carcinoma. High cellularity, diffusely infiltrative solid growth pattern, as well as areas of necrosis and hemorrhage are typical. (**h**) Anaplastic (undifferentiated) thyroid carcinoma at high-power magnification. Primitively looking cell population with brisk mitotic activity (*white arrows*) and occasional multinucleated giant cells (*yellow arrow*) with overall sarcomatoid appearance underscore the loss of differentiation in this tumor type. (**i**) C-cell hyperplasia is barely visible on conventional HE-stain. (**j**) Immunohistochemistry for calcitonin reveals numerous C-cells occupying entire follicles or of parts of them (same location as in **i**). Note the intrafollicular location of the C-cells (the term "parafollicular cells" is actually a misnomer). (**k**) Medullary thyroid carcinoma. This tumor can show quite different growth patterns and cytologic features. Coarse "salt-and-pepper" chromatin is one of the most consistent features aiding the correct diagnosis. (**l**) Immunohistochemical stain for calcitonin is strongly positive in virtually all medullary carcinomas and is obligatory for definitive diagnosis. (**m**) Amyloid deposits in the tumor stroma are often seen and are positively stained with *congo red*. (**n**) Amyloid stained with *congo red* is birefringent and therefore shines *apple-green* in polarized light (same location as in **m**). This feature is essential in distinguishing true amyloid deposits from its mimics (e.g., hyalinized collagenous stroma) (color figure online)

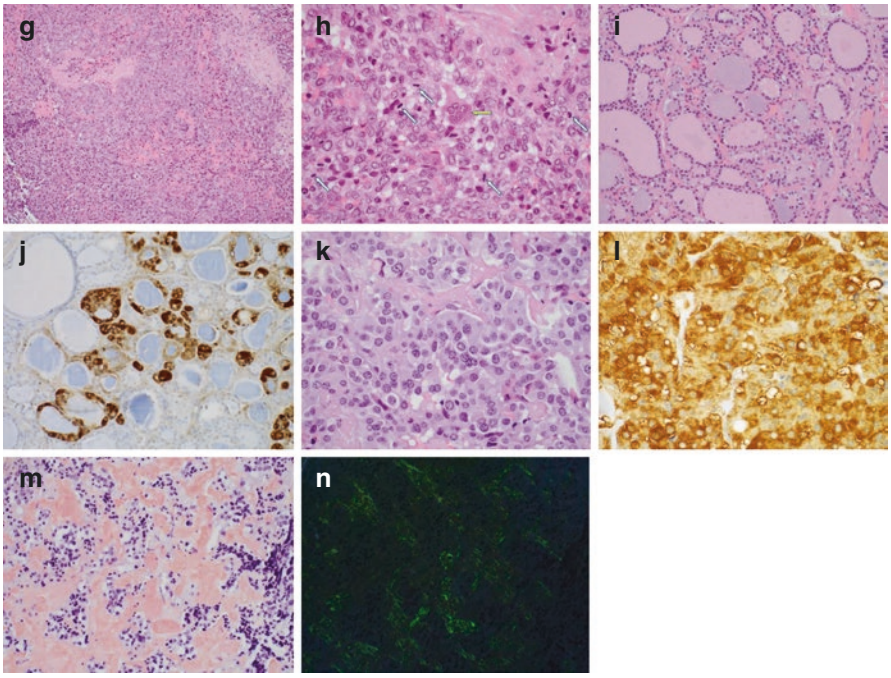

Fig. 10.1 (continued)

10.2 Epidemiology and Pathophysiology of Thyroid Cancer

Worldwide thyroid cancer accounts for less than 2% of all cancers diagnosed [7]. The distribution between female and male patients varies from country to country but is always higher in females, with an average ratio of 3:1 [8]. Even if thyroid cancer has a low prevalence and is associated with more than 95% survival with a very good survival rate, it must not be treated lightly. The incidence for thyroid cancer is significantly rising, including all tumor sizes and stages [9].

The majority of thyroid tumors originate from follicular cells as epithelial tumor. These lesions can further be divided in papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), or anaplastic thyroid carcinoma (ATC). Tumors that arise from C-cells are of the category medullary thyroid carcinoma (MTC) [10]. Eighty-five to 90% of thyroid cancer cases present as PTC, 5–10% as FTC, and about 2% as MTC [10]. Further subtypes include carcinomas of mixed origin, squamous cell carcinoma, mucinous carcinoma, carcinosarcoma, and other less frequent variants [11]. Representative pictures of the various types of thyroid cancer are shown in Fig. 10.1.

A number of genetic alterations accompany the molecular carcinogenesis of the different tumor variants. A summary of these alterations and the associated

signaling pathways, as well as a discussion of how they orchestrate tumorigenesis in the thyroid, is presented below.

10.3 MAP Kinase and PI3-Kinase Signaling Cascades

From all occurring mutations, most affect the signaling pathways of (mitogen-activated protein) MAP kinases and (phosphoinositide-3) PI3-kinase. Among these, we reckon mutations in the genes of *BRAF*, *HRAS*, *KRAS*, or *NRAS*, translocations of *RET*, as well as *PTEN* gene mutations or deletions.

The RAS/RAF/MEK/ERK pathway is among the most essential pathways of inter- and intracellular signaling and of vital importance in signal transductions regulating survival, growth, differentiation, migration, and cell-cell interactions [12]. The best described MAP kinases include ERK proteins, JNK, and p38 [13]. Each of them contains a three-tiered kinase cascade of a MAP kinase (MAPK), a MAP kinase kinase (MAPKK), and a MAP kinase kinase kinase (MAPKKK). Signals are received at the surface of the cell by different receptor molecules, activating signaling modules in the cells interior by phosphorylation events. The most intensively studied pathway is RAS/RAF/MEK/ERK (extracellular signal-regulated kinase) cascade. RAS is recruited to the intracellular domain of a common receptor tyrosine kinase (RTK), which has undergone dimerization upon binding of an extracellular stimulus. When activated by GTP at the cell membrane, RAS can mediate RAF activation. These kinases phosphorylate MEK kinases, and these are capable of activating ERK. ERK can target a wide portfolio of substrates in essentially every cellular compartment to regulate the appropriate cellular response [14].

There are three isoforms of the RAS protein, which occur in the thyroid HRAS, KRAS, and NRAS. Mutations may occur in any of the three, but most publications name *NRAS*′s codon 61 as the site being altered most often in case of *RAS* mutation [14]. Codon 61 encodes for the autocatalytic GTPase of RAS, and an alteration therefore could transform the RAS molecule to a constantly signaling trigger toward over- and mis-reaction. Another site that is frequently mutated is the GTP binding site, encoded in codons 12/13, which increases binding affinity or even locks GTP in the activating position. Mutant activation of RAS has been shown in vivo as well as in vitro to be able to induce thyroid neoplasia. Mutations are understood to occur at early stages, when cells are still well differentiated. At this stage, they were also identified as mutually exclusive with other genetic alterations in FTC and PTC. Mutations of one *RAS* isoform occur in up to 20% of PTC and 50% of FTC cases [14]. *RAS* mutations can also occur in follicular adenomas of the thyroid gland. These lesions are rather frequent and benign, but can also present a precursor lesion of FTC [14].

A point mutation at position 1799 from T to A in the *BRAF* gene is the most common gene mutation in PTC and may also occur in ATC. This mutation, also called V600E, results in constitutive activation of the serine/threonine kinase. Generally, the average rate of *BRAF* mutation in PTC and ATC is approximately 44% and 24%, respectively [15]. Especially the tall cell variant of PTC is characterized by $BRAF^{V600E}$, where up to 100% of cases bear the mutation. On the

contrary, *BRAF*^{V600E} is rarely found in the follicular variant of PTC [16]. Increasing patients' age was identified as predisposing factor to sporadic *BRAF* mutation [17]. *BRAF*^{V600E} is associated with poor clinical outcome, aggressive pathological features, and higher recurrence rate [18]. Furthermore, the mutation is suggested to influence a patient's sensitivity to radiotherapy, as *BRAF*^{V600E} is described to cause a loss of avidity to radioiodine [19].

Charles et al. [20] have shown that *BRAF*^{V600E} is the driver mutation in adult PTC thyroid carcinogenesis, rather than mutations of *KRAS* (especially *KRAS*^{G12D}). A more aggressive mouse model was introduced by McFadden et al., who confirmed that *BRAF*^{V600E} is sufficient to initiate PTC in adult mice thyroid, but also showed that in advanced ATC, *BRAF*^{V600E} is not sufficient as therapeutic target. A combinatorial approach of MAPK pathway targeting by administration of MEK, as well as BRAF inhibitors, showed improved response rates in their respective mouse model. Furthermore, they could show that progression of *BRAF*^{V600E}-positive thyroid cancer to ATC is facilitated by loss of p53 [21].

As mentioned above, besides MAPK pathway regulators, effector proteins of the PI3K signaling cascade are frequently affected by the processes of molecular carcinogenesis in thyroid cancer. The PI3K cascade can be triggered by RAS signaling or other initiators like tyrosine kinase receptors or G protein-coupled receptors. PI3K catalyzes the transition of phosphatidylinositol bisphosphate (PIP2) to phosphatidylinositol trisphosphate (PIP3). PTEN antagonizes this reaction by dephosphorylating and inactivating PIP3. PIP3 in its phosphorylated form is capable of activating Akt. This serine-threonine kinase has multiple targets, of which TSC2, leading to downstream activation of mTOR, is the most popular and influential on protein synthesis and cell cycle progression. Mutations in *PI3K*, *PTEN*, and *AKT* itself are rare in early stages of thyroid cancer and frequently associated with disease progression or even metastasis [22]. In this context, it has to be noted that *PTEN* mutation and especially its deletion occur in approximately 30% of FTC cases. This is frequently in connection with Cowden's syndrome, a cancer predisposition syndrome with characteristic germ line mutation of *PTEN*. Besides mutation and deletion alterations, *PTEN* expression may also be modified by promoter hypermethylation, which occurs in FTC and ATC cases [23].

10.4 Gene Translocations and Fusions

Among the cell surface tyrosine kinase receptors that transduce extracellular signals to downstream signaling cascades as introduced above, the transmembrane protein rearranged during transfection (RET) is an important player in thyroid carcinogenesis. Mehlen and Bredesen [24] reported RET as belonging to the group of so-called dependence receptors. Unbound, RET possesses proapoptotic activity that is interrupted as soon as a ligand binds to the extracellular domain. This effect is the basis for the concept that *RET*-expressing cells might be controlled in a way that their growth and survival is limited to ligand co-localization. Effects on the development of cancer or other diseases are not elucidated in full detail [25]. Still, the cancer-associated mutant *RET*^{C634R} has no cleavage-dependent proapoptotic effect. In

thyroid cancer, the most frequent alteration of *RET* is a genetic disruption of the gene leading to translocation and gene fusion with various heterologous genes. The resulting chimeric oncogenes are termed *RET/PTC* [25]. Prevalence of *RET/PTC* is highly dependent on the cohort under investigation and may range from 25% up to 70% in patient groups, including pediatric patients and individuals with high radioiodine isotope load [26]. Generally, *RET/PTC* formation enables constitutive RET kinase dimerization, activation independent of ligand binding, and autophosphorylation, which leads to steady downstream signaling.

Mouse models harboring *RET/PTC* rearrangements have shown a sufficiency to initiate thyroid carcinogenesis. Corresponding mouse lines were generated by two independent groups, where the transgene was expressed under different promoters and at varying copy numbers. All *RET/PTC* mice developed PTC, thus, at copy-number-dependent rates [27, 28].

RET/PTC rearrangement, other than *BRAF* mutation, which is a distinct tumor indicator, was also reported in benign nodules or healthy tissue surrounding tumor tissues [29]. Various studies report a rate of *RET/PTC* rearrangement in 13–15% of benign nodules. The rate is even higher in individuals with a history of irradiation. The rate of 52.4% *RET/PTC* rearrangement in post-Chernobyl benign nodules is almost identical to the rate of *RET/PTC* rearrangement in PTC [30, 31].

A further gene translocation occurring in up to 60% of FTC cases is the paired box 8 (PAX8)/peroxisome proliferator-activated receptor gamma (*PPARG*) gene fusion. It may also occur in approximately 30% of follicular variant PTC cases [32]. PAX8 plays an essential role in the terminal differentiation steps of thyrocyte development and, unlike other members of the *PAX* gene family, is a key regulator of terminally differentiated gene expression, including the sodium iodide symporter, thyroglobulin, and the TSH receptor [33]. It is discussed controversially how oncogenic the gene fusion *PAX8/PPARG* actually is. On the one hand, it was shown that the resulting fusion protein can act as a suppressor on PPARG-driven gene expression and thereby executes antiapoptotic features. On the other hand, it was shown that the fusion protein can disrupt PAX8 as a transcription factor and deregulate the expression of thyroid-specific genes [33].

10.5 Further Influential Molecular Alterations Contributing to Thyroid Carcinogenesis

Besides the genetic variations introduced above, this section summarizes further frequent and important alterations in thyroid cancer.

With a low involvement in the development but high impact on tumor progression, mutations on *p53* have to be mentioned here. P53 is the most commonly mutated tumor suppressor gene in all human cancers and associates with bad prognosis. Also in thyroid cancer, mutation of *p53* marks a malignant progression of an individual cancer. In accordance, *p53* mutation is detectable in 70–80% of ATC cases [34].

A similar frequency of 70–80% in ATC and 25% in poorly differentiated thyroid cancer is witnessed for mutations of the *CTNNB1* gene. *CTNNB1* encoding for β-catenin is a key regulator in the WNT pathway. Aberrant Wnt signaling is a

hallmark of epithelial tumors' developmental phase. The family of Wnt proteins has a physiological role in embryonic development, controlling cell proliferation, cell fate specification, tissue patterning, and cell polarity. Later, in adult tissue, the proteins are involved in tissue homeostasis, as they control cell proliferation, stem cell activation, and self-renewal. Wnt proteins can execute their targeted signaling via three different pathways, including a canonical β-catenin-dependent and two β-catenin-independent pathways. The latter noncanonical pathways are a calcium pathway and planar cell polarity (PCP) signaling [35]. The canonical Wnt signaling, which is dependent on β-catenin, is extensively studied and known to contribute to cancer development and progression in various tumor entities [36]. This signaling cascade is triggered by extracellular binding of a Wnt protein to a frizzled receptor (Fzd) which consequently leads to recruitment of Axin to the cell membrane. This releases Axin's former interaction partners, including β-catenin, which will accumulate in the cytoplasm and enter the nucleus. Nuclear β-catenin acts as a transcription factor for Wnt target genes, like cyclin D1, c-Myc, and further potent regulators of cell proliferation [37]. In the absence of Wnt, β-catenin is tightly bound to E-cadherin in adherens junctions. For many years, Wnt pathway dysregulation was associated solely with ATC and as a feature of aggressive thyroid carcinoma. Throughout the last decade, evidence has been found that altered Wnt signaling is influential also on early stages of thyroid carcinogenesis. Indicators thereof are elevated levels of Wnt family member Wnt5a in FTC and PTC or the stabilization of cytoplasmic β-catenin by RET/PTC [38, 39].

10.6 Epigenetic Modifications in Thyroid Cancer Development and Progression

There are two main fields of epigenetic regulation reported to influence thyroid carcinogenesis. These are, on the one hand, microRNAs (miRNAs) and, on the other hand, aberrant methylation events.

MiRNAs are small noncoding RNAs of approximately 22 nucleotides length that bind to multiple mRNAs, initiating translational repression by cleavage of target mRNA. As for thyroid cancer, it was very early discovered that miRNAs might play an important role in PTC development and progression, as a set of miRNAs could be identified as significantly overexpressed, whereas, in general, cancers are associated with a global under-expression of miRNAs [40]. The most consistently overexpressed miRNAs reported in PTC are miR-221, miR-222, and miR-146b [41].

DNA methylation at cytosines, especially in CpG islands, is a powerful regulator of gene expression. Hypomethylation can cause genetic instability and activation of proto-oncogenes; on the contrary, hypermethylation may silence a gene and is frequently identified as causative for tumor suppressor gene downregulation. Affected tumor suppressor genes in thyroid cancer include *PTEN, RASSF1A, TIMP3, SLC5A8, DAPK, RAPβ2*, and *RAP1GAP* [42]. Aberrant DNA methylation of these genes is detectable in up to 100% of tumors analyzed.

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