Molecular Pathogenesis of Thyroid Cancer and Oncogenes in Thyroid Cancer

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Oncogenes and Tumor Suppressor Genes

Throughout their lifespan, somatic cells can be thought of as progressing through three overlapping transitional stages. Stem cells initially proliferate by undergoing repetitive cell division, causing a rapid expansion of immature tissue mass. Subsequently, these cells differentiate into mature cells that deliver the functions characteristic of their particular phenotype. Later, they grow senescent and undergo programmed cell death or apoptosis. Tumor development (or neoplasia) results from stimuli that augment cellular proliferation or impair cell differentiation and/or apoptosis. A diverse set of signaling and effector proteins is involved in the precise regulation of this complex series of events. Mutations in the genes encoding these proteins have been found to underlie the majority of human malignancies [143]. Genes that encode the proteins promoting normal cell proliferation are called proto-oncogenes. Proto-oncogenes develop activating or gain-of-function mutations that result in the production of proteins that are qualitatively overactive or quantitatively excessive and thereby promote over-robust cellular proliferation. These mutated proto-oncogenes are known as oncogenes [25, 36, 50, 61, 91, 93, 143]. Oncogene mutations tend to be dominantly expressed and thus become clinically apparent in the heterozygous state.

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Tumor suppressor genes [50, 70, 89, 102, 142] encode the proteins that serve to restrain excessive cellular proliferation or promote cell differentiation and/or apoptosis. Inactivating or loss-of-function mutations of these tumor suppressor genes can also lead to neoplasia; these tend to be recessive and therefore are clinically consequential only when present in the homozygous or compound heterozygous state.

Cells undergoing unregulated proliferation because of an activated oncogene or inactivated tumor suppressor gene are considered to be transformed. Cancer-causing mutations may be either somatic or germline. Somatic mutations are those that develop in a single cell at any time in life after fertilization. Through survival advantage conferred by the mutation, the transformed cell expands monoclonally into a solitary tumor mass that may eventually invade or metastasize. In contrast, germline mutations originate in a parent and are passed to offspring through a germ cell. Affected offspring have the mutation present diffusely and may thus be susceptible to the development of multiple tumors within a given organ or susceptible to tumors in multiple organs throughout the body. Most known inherited cancer syndromes result from germline mutations in tumor suppressor genes. Accordingly, individuals are born heterozygous at a critical locus but are initially unaffected because of the normal gene at the homologous locus. However, if a somatic mutation later in life inactivates the normal homologous locus, the individual is rendered unable to make any normal suppressor protein and begins to develop cancer.

The complex system that regulates cellular proliferation, differentiation, and apoptosis has many checks and balances. Although a single genetic mutation may initially transform a cell permitting the monoclonal expansion of its progeny, it is unlikely that a single mutation alone could result in the development of highly malignant tumor behavior. Yet, it appears that the unregulated proliferation of a transformed cell predisposes it to develop additional mutations. These, in turn, provide further selective survival advantages by promoting

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ever-accelerating cell proliferation, tissue invasion, and distant metastases. Indeed, experimental evidence indicates that multiple activated oncogenes and inactivated tumor suppressor genes are often found in highly malignant and metastatic tumors [8, 141].

Similar to other cancer types, thyroid cancer initiation and progression occurs through accumulation of genetic and epigenetic alterations, including activating and inactivating somatic and germline mutations in proto-oncogenes and tumor suppressor genes. Somatic mutations in follicular cells occur early in carcinogenesis and trigger malignant transformation.

Pathways Affected in Thyroid Neoplasia

Molecular defects resulting in oncogenic transformation frequently occur in pathways controlling cell proliferation and survival. Thyroid cancer mutations develop most often in genes encoding components of the MAPK/ERK and PI3K/AKT pathways (Fig. 3.1). The MAPK/ERK pathway is activated in response to a diverse array of stimuli, such as mitogens, growth factors, and pro-inflammatory cytokines, and it regulates cell proliferation, differentiation, apoptosis, and survival. Genetic alterations in the MAPK/ERK pathway result in constitutive activation of signaling and can therefore be pro-tumorigenic.

The PI3K/AKT pathway promotes cell cycle progression (reviewed in [118]) and is a key regulator of survival during cellular stress. Activation of growth factor receptor protein tyrosine kinases results in autophosphorylation of tyrosine residues, PI3K recruitment to the cell membrane, and allosteric activation of the catalytic subunit encoded by a gene *PIK3CA*. This leads to production of the second messenger phosphatidylinositol-3,4,5-triphosphate (PIP_3) which then recruits a subset of signaling proteins, including protein kinase AKT, to the membrane. AKT inactivates pro-apoptotic factors such as BAD and procaspase-9. AKT positively regulates G1/S cell cycle progression acting through mTOR and increased cyclin D1 activity. AKT also controls the IκB kinase complex of the pro-survival NFκB pathway. Both MAPK/ERK and PI3K/AKT pathways converge at the level of RAS.

Thyroid cancer develops as a result of mutations in growth factor receptor tyrosine kinases signaling through MAPK/ERK and PI3K/AKT. Mutations in RET, TRK, and ALK tyrosine kinases are discussed in detail below. Since these receptors are expressed in normal neuroendocrine parafollicular C-cells, but not in thyroid follicular cells, gene fusions altering their cell-specific expression pattern are necessary to

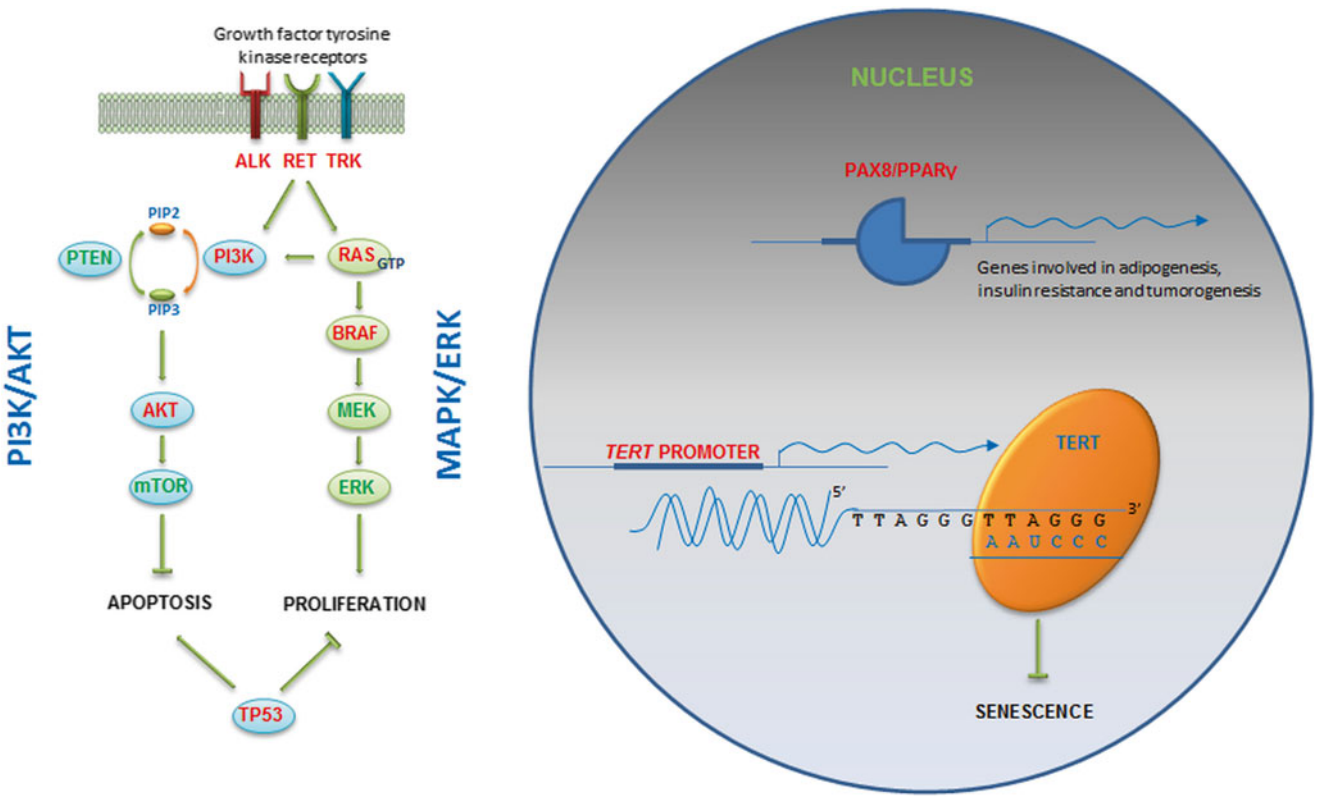

Fig. 3.1 Pathways affected in thyroid cancer. Mutated proteins and genes are highlighted in *red*

cause transformation in thyroid cancer types other than medullary thyroid cancer (MTC).

The *TERT* gene encodes the catalytic subunit of telomerase, the specialized DNA polymerase that lengthens telomeres. Chromosomes are capped by telomeres that replicate incompletely and thus get shorter with each division. Telomere shortening causes replicative senescence, which blocks cell division. Telomerase activity, which prevents telomere shortening and subsequent apoptosis, is absent in non-immortalized cells, including normal follicular cells, but is expressed in the majority of cancers. *TERT* mutations are unique as genetic sequence alterations occur in the promoter region but not in the coding sequence of the gene.

Papillary Thyroid Carcinoma: *BRAF, PIK3CA, RET/PTC, NTRK1*, and *TERT* Mutations

The majority of papillary thyroid carcinomas (PTC) are characterized by mutations in genes for components of the MAPK/ERK pathway: *RET/PTC* and *BRAF*. The *BRAF* gene encodes an intracellular serine-threonine kinase that phosphorylates and activates downstream targets of MAPK/ERK signaling such as MEK. *BRAF* gene mutations are the most prevalent genetic alterations observed in thyroid cancer. A point mutation at nucleotide 1,799 produces a change from a valine to a glutamine at amino acid residue 600 (*BRAF V600E*) of the resulting protein that leads to constitutive BRAF dimerization and chronic activation of the MAPK pathway [27, 83]. Other *BRAF* gene mutations such as *K601E* [138], small in-frame deletions and insertions near codon 600 [20, 21, 74, 137] and even *AKAP9/BRAF* gene fusions [23], have been described, but constitute less than 2 % of all mutations of this gene in a sporadic thyroid cancer. Mutations in *BRAF* have been shown to be unique to PTC and advanced forms of thyroid cancer, poorly differentiated thyroid carcinoma (PDTC), and anaplastic thyroid carcinoma (ATC) that originate from PTC [54, 83, 114]. *BRAF* mutations are found in approximately 45 % of all PTC [5, 27, 54, 83, 114, 116, 135, 150].

Strong evidence indicates that a *BRAF V600E* mutation is associated with a higher risk of recurrence (25–30 % and 10–12 % overall risk for BRAF-mutated and wild-type PTC, respectively). Mutation is linked to higher-risk phenotypes, presence of lymph node metastases, and extrathyroidal extension [5, 97]. However, BRAF mutation is not independently associated with disease mortality and most of the attributable risk is conveyed by histologic analysis. Equally important, BRAF mutation does not affect the prognosis of papillary microcarcinomas, which are associated with 1–2 % risk of recurrence [139, 149]. In addition to promoting tumor cell proliferation/transformation though persistent activation of the MAPK/ERK pathway, a number of mechanisms have been suggested to explain the more aggressive nature of tumors harboring a *BRAF V600E* mutation, such as reduced expression of immune/inflammatory response genes and escape from immune surveillance [134] and silencing of the sodium-iodine symporter gene [154]. Currently, *BRAF* gene testing is not recommended for initial risk stratification in differentiated thyroid cancer, but the field of genetic biomarkers is rapidly evolving.

RET and TRK are receptor tyrosine kinases that signal through MAPK/ERK and PI3K/AKT pathways. Both proteins are present in neuroendocrine C cells but are not expressed in normal thyroid follicular cells. Therefore, activating point mutations in these genes are found in MTC but do not occur in thyroid cancers of follicular cell origin. Instead, chromosomal rearrangements resulting in the formation of chimeric genes consisting of a promoter and a 5′ prime segment of a gene constitutively expressed in follicular cells, and a 3′ segment encoding a kinase domain of RET or TRK occur in follicular cell-derived cancers [66]. Resulting chimeric proteins lead to constitutive stimulation of MAPK/ERK signaling. The most common (>90 %) fusion partners of *RET* are *CCDC6* and *NCOA4* located on the same chromosome 10 producing fusion genes known as *RET*/*PTC1* and *RET*/*PTC3* [66, 129] (Table 3.1). The other known *RET* rearrangements (Table 3.1) are interchromosomal. *RET* fusion partner genes encode ubiquitously expressed proteins that contain coiled-coil domains responsible for ligand-independent dimerization of the hybrid protein and constitutive activation of the tyrosine kinase domain of RET. Clonal rearrangements involving the *RET* gene are the second most common genetic alteration seen in PTC, following *BRAF* mutation, and are seen in 10–20 % of patients [83, 128].

TRK oncogenes arise from chromosomal rearrangements involving the *NTRK1* gene. This gene, located on chromosome 1, encodes the high affinity receptor for nerve growth factor that is important for neuronal differentiation and maturation. *TRK* oncogenes contain 5′ sequences from the *TPM3* and *TPR* genes on chromosome 1 and the *TFG* gene on chromosome 3 (Table 3.1). All TRK oncoproteins retain the NTRK1 tyrosine-kinase domain, five tyrosine residues crucial for NTRK1 activity, and are ectopically expressed in thyrocytes. Constitutive dimerization further contributes to upregulated kinase activity [64]. *NTRK1* rearrangements are less frequent in PTC compared to *RET* gene fusions.

Alterations of PI3K signaling in thyroid cancer occur in a number of ways. Mutations affecting genes for the catalytic α-subunit of the kinase PIK3CA [116], AKT (PIP_3 target), and PTEN (PIP_3 phosphatase) are prevalent in undifferentiated forms of thyroid cancer [29, 56, 73, 116, 122, 126]. Genetic alterations of PI3K/AKT pathway are frequently coexisting with mutations in other genes driving malignant transformation, suggesting that these are late events in cancer progression.

Table 3.1 Gene fusions in thyroid cancer.

Rearrangement	Fusion partner	Fusion partner location	Function of the protein encoded by fusion partner gene	Type of thyroid cancer	References
RET/PTC1	*CCDC6*	chr 10	Cellular response to DNA damage	PTC	[66, 117]
RET/PTC2	PRKAR1A	chr 17	Regulatory subunit of type I protein kinase A	PTC	[10]
RET/PTC3	*NCOA4*	chr 10	Ligand-dependent androgen receptor coactivator	PTC, radiation-associated PTC	[105, 129]
RET/PTC4	*NCOA4*	chr 10	Ligand-dependent androgen receptor coactivator	PTC	[52]
RET/PTC5	*GOLGA5*	chr 14	Coiled-coil protein on the Golgi surface	PTC	[85]
RET/PTC6	*TRIM24*	chr 7	Binds chromatin and estrogen receptor to activate estrogen-dependent genes associated with cellular proliferation and tumor development	PTC	[88]
RET/PTC7	*TRIM33*	chr 1	Transcriptional regulator, binding partner of SMAD2 and SMAD3 as a part of canonical TGF-beta pathway	PTC	[88]
RET/PTC8	*KTN1*	chr 14	Anchors elongation factor-1-delta to the endoplasmic reticulum membrane	PTC	[125]
RET/PTC9	*RFG9*	chr 18	Unknown	PTC	[86]
PCM1-RET	*PCM1*	chr 8	Component of centriolar satellites	PTC	[28]
RFP-RET	*TRIM27*	chr 6	Transcription corepressor	PTC	[124]
ELKS-RET	*RAB6IP2*	chr 12	Regulatory subunit of the IKK complex	PTC	[110]
HOOK3-RET	*HOOK3*	chr 8	Microtubule protein	PTC	[22]
TRK	*TPM3*	chr 1	Tropomyosin, a component of the thin filaments of the sarcomere	PTC	[14]
TRK-T1, *TRK-T2*	*TPR*	chr 1	Protein involved in mRNA export	PTC	[63, 65]
TRK-T3	*TFG*	chr 3	Plays role in the function of the endoplasmic reticulum and its associated microtubules	PTC	[62]
PAX8-PPARγ	*PAX8*	chr 2	Thyroid-specific transcription factor essential for thyroid development	FTC, fvPTC	[90]
ETV6-NTRK3	*ETV6*	chr 12	Ets family transcription factor	Radiation-associated PTC	[95]
CREB3L2- PPARγ	*CREB3L2*	chr 7	Member of bZIP family of transcription factors	FTC	[99]
AKAP9-BRAF	*AKAP9*	chr 7	Scaffolding protein for protein kinases and phosphatases	Radiation-associated PTC	[23]
STRN-ALK	*STRN*	chr 2	Ca2+-dependent scaffold protein	ATC, PDTC, PTC	[82]
EML4-ALK	*EML4*	chr 2	Unknown	ATC, PDTC, PTC, radiation-associated PTC	[69, 82]

TERT promoter mutations are relatively infrequent in PTC.

Mutations in *BRAF, RET*, and *RAS* genes(follicular variant of PTC (fvPTC) and follicular thyroid cancer (FTC)) are mutually exclusive, consistent with their roles as driver mutations [83, 135]. In the ThyroSeq cohort, the frequency of the *BRAF V600E* allele was >50 %, supporting its role as a major clonal driver [116]. Alterations of other signaling cascades may rarely coexist with *BRAF* gene mutations. This has been described for *PIK3CA* [73], *TP53* [116], and *TERT* genes [92, 98].

Follicular Variant of Papillary Thyroid Carcinoma: *RAS* Mutations and *PAX8/PPARγ* Rearrangements

The molecular profile of fvPTC is different from classic and tall cell variants, more closely resembling that of the follicular adenoma/carcinoma group of tumors. *RAS* is the most prevalent mutation type in fvPTC [1, 116]. The *PAX8/PPARγ* rearrangement has also been found, though less frequently, in encapsulated fvPTC [123]. *BRAF V600E* mutations are rare in fvPTC [26, 51, 114, 138, 148] although, interestingly, nonclassic mutations in *BRAF* (K601E and small deletion/insertions) are associated with fvPTC [5, 138].

Radiation-Associated Papillary Thyroid Carcinoma: Gene Fusions

Thyroid cancer caused by exposure to ionizing radiation, either therapeutic or as a consequence of nuclear plant accidents, has a distinct genetic background with a greater prevalence of genetic rearrangements. This is plausibly explained by the ability of ionizing radiation to cause double-stranded DNA breaks, facilitating the formation of fusion genes.

Up to 80 % of PTC in patients exposed to ionizing radiation have *RET/PTC* rearrangements (most frequently *RET/PTC3*) [12, 53, 87, 111, 119, 121]. Other fusion genes associated with radiation-induced thyroid cancer are *AKAP9-BRAF* [23], *ETV6-NTRK3* [95], *CREB3L2-PPARG*, and *AGK-BRAF* [121]. *EML4-ALK* gene rearrangements have been found to occur frequently in PTC among atomic bomb survivors [69]; however, this finding was not reproduced in another study [95].

Rearrangement-positive PTCs are associated with greater I131 exposure and possibly iodine deficiency [68, 94, 95]. In contrast, *BRAF* and *RAS* point mutation frequency is negatively correlated with radiation dose [94, 136].

Follicular Adenomas and Follicular Carcinomas: *RAS* and *PAX/PPARγ* Mutations

Three distinct *RAS* genes are known, *HRAS*, *KRAS*, and *NRAS*; these genes encode 21-kDa G-proteins that transmit signals from membrane receptors to the mitogen-activated protein kinase (MAPK) and PI3K/AKT pathways. G-proteins, including RAS, are located at the inner surface of the cell membrane and are bound to GDP in an inactive state. Ligand binding at the corresponding membrane receptor results in activation of RAS through binding to GTP (with the help of guanine nucleotide exchange factor, GEF) and downstream signaling. Intrinsic GTPase activity of RAS is responsible for protein inactivation and signal transduction termination. *RAS* gene point mutations that occur in codons 12, 13, and 61 reduce GTPase activity of the RAS protein, constitutively activating downstream signaling cascades.

RAS mutations have been found in 40–50 % of both follicular adenomas and FTC [44, 96, 115]. Because of their presence in both benign and malignant thyroid lesions, it has been suggested that *RAS* mutations alone may not be sufficient for malignant transformation of thyroid cells but may be an early event in thyroid tumorigenesis predisposing to acquisition of additional genetic or epigenetic alterations that lead to a fully transformed phenotype [115]. This theory is supported by animal studies in transgenic mice with thyroid-specific expression of mutant *KRAS* that develop benign thyroid nodules and follicular adenomas [127].

The *PAX8/PPARγ* fusion protein is created by a t(2; 3) (q13;p25) chromosomal translocation [90, 120]. *PAX8* encodes a transcription factor essential for thyroid development that drives the expression of thyroid-specific genes such as thyroid peroxidase and thyroglobulin. Peroxisome proliferator-activated receptor γ (PPARγ) is a ubiquitously expressed transcription factor that has a role in glucose homeostasis, lipid metabolism, inflammation [3], and tumorigenesis [132].

This *PAX8/PPARγ* rearrangement in thyroid cancer results in an overexpression of a chimeric transcription factor [49]. It was suggested that PAX8/PPARγ functions as a dominant negative suppressor of wild-type PPARγ activities [90], but the exact molecular mechanism of malignant transformation remains to be uncovered. This hypothesis is challenged by a finding that depletion of PPARγ resulted in decreased cell growth of ATC tumors in an animal model [147].

The *PAX8/PPARγ* rearrangement is the second most common genetic alteration in FTC, found in 30–35 % of tumors, and is associated with a more invasive phenotype [37, 49, 101, 113, 115]. An alternative *PPARγ* fusion, *CREB3L2/PPARγ*, has been found in a very small fraction (<3 %) of FTC [99]. With rare exceptions, a *PAX8/PPARγ* mutation is mutually exclusive with a *RAS* mutation.

In mouse model of thyroid cancer caused by the *Pax8/Pparγ* rearrangement, the PPARγ agonist pioglitazone triggers redifferentiation of cancer cells into adipocytes [33]. This finding translated into a clinical trial (NCT01655719) testing the use of pioglitazone for the management of advanced FTC and fvPTC carrying the *PAX8/PPARγ* fusion.

PAX8/PPARγ mutations also occur in benign follicular adenomas [101, 113, 115] but at a lower frequency.

Hurthle cell adenomas and carcinomas have a very low frequency of either *RAS* mutations or *PAX8-PPARγ* rearrangements, suggesting that these tumors are a distinct type of thyroid neoplasm [49, 115].

TERT promoter mutations are more frequent in FTC compared to PTC and occur in 17–36 % of cases [98, 103]. The causative role of *TERT* mutations in development and progression of differentiated thyroid cancer has yet to be proven.

Medullary Thyroid Carcinoma: *RET* Point Mutations

RET is a tyrosine kinase receptor for the glial-derived neurotrophic factor (GDNF) family of ligands: GDNF, neurturin, artemin, and persephin [4, 80]. It is expressed in neuroendocrine calcitonin-producing parafollicular C cells of the thyroid but not in follicular cells. Ligand binding results in dimerization of the RET receptor and autophosphorylation of intracellular tyrosine residues that function as docking sites for adaptor proteins. The RET signaling network is very complex; RAS/ERK, PI3K/AKT, STAT3, c-Src, PLCγ, NFκB, JNK, and other pathways are activated depending on which tyrosine residue is phosphorylated or non-tyrosine-dependent mechanism is activated (reviewed in [30, 76, 145]).

MTC occurs as a component of three distinct dominantly inherited cancer syndromes: multiple endocrine neoplasia type 2A (MEN 2A), associated with pheochromocytoma and primary hyperparathyroidism; multiple endocrine neoplasia type 2B (MEN 2B) that presents with pheochromocytomas,

mucosal neuromas, intestinal ganglioneuromas, and functional gastrointestinal disturbances; and familial MTC syndrome (FMTC), in which patients develop MTC only.

In 1993, it was discovered that germline point mutations in the *RET* proto-oncogene cause MEN 2A and MEN 2B as well as FMTC [19, 35, 71, 108].

MEN 2A results from mutations of cysteine codons 609, 611, 618, 620 (exon 10), 630, and 634 (exon 11). Familial MTC is caused by mutations in codons 609, 618, 620 (exon 10), 768, 790, 791 (exon 13), 802, 844 (exon 14), and 891 (exon 15). The genetics of MEN 2B syndrome is less variable with the majority of cases (>95 %) caused by a mutation in codon 918 (exon 16), causing a replacement of methionine with threonine within the catalytic core region of the tyrosine kinase domain [19]. Rarely, MEN 2B is associated with a mutation A883F (exon 15) [59] or double *RET* mutations, such as V804M/Y806C [79] and V804M/S904C [104]. A full list of *RET* mutations and their association with particular syndromes and clinical presentation is available in the 2009 ATA guidelines for the management of MTC [84].

MEN 2A mutations are localized within the cysteine-rich domain of RET and cause ligand-independent dimerization and constitutive kinase activity of the RET protein. MEN 2B mutations that occur within the intracellular tyrosine kinase domain of RET have no effect on receptor dimerization but do cause constitutive activation of intracellular signaling pathways [48]. FMTC mutations affect both cysteine-rich and tyrosine kinase domains.

The existence of clear genotype-phenotype correlations useful for clinical management with respect to screening, surveillance, and prophylaxis has made *RET* genotyping in familial MTC cases a successful application of personalized medicine [41, 84]. For example, patients carrying mutations specific for MEN 2B are considered to have the greatest risk for aggressive MTC; they require prophylactic thyroidectomy as soon as possible within the first year of life and early screening for pheochromocytoma, but not for primary hyperparathyroidism [48, 84]. Early diagnosis followed by an early intervention results in improved outcomes; more than 95 % of patients whose disease was detected at an early stage have remained disease-free [47, 100, 133].

Somatic *RET* mutations are found in approximately 40–78 % of patients with sporadic MTC, occurring sometimes at codons 608, 611, 618, 629, 630, 634, 641, 649, 918, and 922, but most frequently at 918, the codon affected in patients with MEN 2B syndrome [2, 30, 38]. Similar to hereditary MTC syndromes, tumors triggered by somatic codon 918 mutations show more aggressive phenotypes [131, 152, 153]. Somatic *RET* mutations are not consistently distributed within primary tumors and metastases and therefore may occur late in tumor development instead of serving as primary driver events [42, 131].

Mutations in the *RAS* family of small GTPase genes have been identified in sporadic MTC, and *RAS* has been proposed to act as an alternative driver of MTC tumorigenesis [2, 9, 24, 107, 116]. *RET*, *KRAS*, and *HRAS* mutations are mutually exclusive. Exome sequencing has found *RET* or *RAS* mutations in as many as 90 % of sporadic MTC [2].

Poorly Differentiated and Anaplastic Thyroid Carcinoma

The classic model of multistep carcinogenesis suggests that anaplastic carcinomas arise from differentiated carcinomas through accumulated damage to the genome. Most of the mutations described in differentiated cancer (*RET*, *HRAS*, *KRAS*, *NRAS*, *BRAF*, *PIK3CA*, *AKT1*) have also been found in PDTC and ATC, albeit at a varying frequency [116]. PDTC and ATC frequently co-localize with PTC in the same patient, and *BRAF* mutations have been reported in both tumors [7, 114]. This provides molecular evidence supporting the hypothesis that some ATC and PDTC originate from PTC.

While driver mutations in differentiated thyroid cancer are generally mutually exclusive, coexistence of several genetic alterations is common in ATC [116]. For example, *PIK3CA*/*AKT1* mutations found in combination with *BRAF* mutations in dedifferentiated thyroid cancer suggest a synergistic effect of alterations in both pathways for thyroid cancer advancement. *PIK3CA*/*AKT1* mutation status is frequently discordant in lesions originating from primary and advanced metastatic thyroid cancer, suggesting that these mutations occur during tumor progression rather than being primary driver mutations [122].

TERT promoter mutations have been implicated in the progression toward PDTC and ATC. Two mutations, C228T and C250T, have been discovered in the promoter region of the *TERT* gene in thyroid cancer cell lines and tissues. Either of these mutations results in the generation of novel consensus binding sites for ETS transcription factors and causes a several fold increase in *TERT* expression [72, 75]. The frequency of *TERT* promoter mutations is relatively low in differentiated thyroid cancers (9–22 %), but it is much higher in PDTC and ATC (51 %) [92, 103]. *TERT* promoter mutations frequently coexist with mutations in known driver genes, such as *BRAF* and *RAS*, in advanced forms of thyroid cancer. It has been hypothesized that *TERT* promoter mutations occurring in DTC cells harboring driver mutations cause transformation into undifferentiated forms of thyroid cancer. Mechanistically, this makes sense considering that ETS transcription factors are regulated by the MAPK/ERK pathway. This hypothesis remains to be proven experimentally and *TERT* promoter mutations may simply represent a marker of genetic instability, rather than true driver of disease progression.

Mutations in the genes encoding the cell cycle regulator, p53 (*TP53*), and the cell adhesion and Wnt signaling protein, β-catenin (*CTNNB1*), are prevalent in PDTC/ATC but not in differentiated thyroid cancers [32, 34, 46, 55, 57, 78]. *TP53* gene mutations are the most common genetic alterations in ATC [116].

Curiously, *RAS* mutations are prevalent in PDTC but, in contrast to *BRAF* mutations, are associated with the absence of extrathyroidal extension and with longer survival [121].

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase that, like RET and TRK, activates the MAPK/ERK and PI3K/AKT pathways, promoting cell proliferation and survival. *ALK* gene fusions were initially found in anaplastic large-cell non-Hodgkin's lymphomas and are infrequent events in non-small cell lung cancer and inflammatory myofibroblastic tumors. *EML4-ALK* and *STRN-ALK*fusions have been found in thyroid cancer [82]. ALK protein is not expressed in normal thyroid tissue, but the fusion genes are overexpressed in tumors carrying the mutation. *STRN-ALK* fusion results in constitutive autophosphorylation and dimerization of the fusion protein that causes ALK kinase activation and MAPK activation. *STRN-ALK* causes transformation of rat thyroid PCCL3 cells, which causes flank tumors in mice. *ALK* fusions have been found in 1.6 % of PTC, 9 % of PDTC, and 4 % of ATC. The fusion was not found in 36 FTC or in 22 MTC. *ALK* fusions are mutually exclusive with other known thyroid cancer driver mutations.

Point mutations in the tyrosine kinase domain of *ALK* have also been found in ATC [109]. Point mutations resulted in an upregulated kinase activity of ALK, increased phosphorylation of ERK and AKT, and promoted cell transformation. The significance of this finding will depend on whether ALK protein is expressed in ATC.

Clinical Applications

Diagnosis

Cytology examination of fine needle aspiration (FNA) samples classifies 70–75 % of thyroid nodules as benign or malignant with great accuracy. The remaining samples are labeled as indeterminate or inadequate, leading to repeated invasive testing and/or unnecessary diagnostic surgery. Molecular testing was developed as an add-on to FNA when cytology analysis was indeterminate. The best molecular test to exclude malignancy, an ideal "rule-out" test, would have a sensitivity and negative predictive value similar to a benign cytologic diagnosis (94–97 %) [11] and would be most useful for the purpose of avoiding unnecessary surgery for benign thyroid nodules. An ideal "rule-in" test would have a specificity and positive predictive value similar to a malignant cytologic diagnosis (97–99 %).

BRAF V600E mutation positivity has very high specificity (>99 %) and excellent positive predictive value for the diagnosis of thyroid cancer [81]. However, a *BRAF* mutation is only detected in a fraction of malignant lesions, and therefore the sensitivity and negative predictive value of the test is poor. An improved sensitivity was achieved by combining the most frequent driver mutations in a test panel [16, 112]. This panel is now commercially available under the name ThyGenX (Interpace Diagnostics) and includes mutations in the *BRAF*, *HRAS*, *NRAS*, and *KRAS* genes as well as the *RET*/*PTC1*, *RET*/*PTC3*, and *PAX8*/*PPARγ* translocations. A similar test is offered by Quest Diagnostics. While testing for the panel of oncogene driver mutations has improved the presurgical diagnosis of thyroid cancer, its sensitivity (~75 %) is not sufficient to confidently rule out malignancy in thyroid nodules with indeterminate cytology. This is particularly true for Bethesda categories with a low prevalence of malignancy (such as category III, atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS)). However, greater specificity and PPV of oncogene mutation testing together with the prognostic value of certain mutations (aggressive behavior of *BRAF V600E* positive tumors, or tumors with more than one genetic alteration; indolent clinical course of fvPTC due to RAS mutations) may assist in deciding on the extent of the surgery (near total thyroidectomy vs. lobectomy in patients with cytology suspicious for PTC) or decision to do prophylactic central neck dissection.

The development of next-generation sequencing technology has made The Cancer Genome Atlas (TCGA) project feasible and has led to significant advances in the field of thyroid cancer genomics. A number of new likely driver mutations have been discovered, increasing the percentage of tumors with known genetic causes to 93 % (Giordano, personal communication). While probing for more driver mutations will no doubt improve the sensitivity of thyroid cancer molecular testing, it will come at the cost of a greater number of false-positive results since some mutations (such as *RAS* and *PAX8*/*PPARγ*) are found in benign lesions as well [37, 101, 115].

Another methodological challenge comes from the varying sensitivity of methods used for detection of mutated genes. Ultrasensitive detection methods are capable of identifying mutated alleles present at low frequency (non-clonal alleles). This problem was systematically studied for *RET*/*PTC* rearrangements, and high-sensitivity RT-PCR was shown to detect non-clonal rearrangements [155]. Highly sensitive methods discovered *RET*/*PTC* rearrangements in benign thyroid nodules and non-neoplastic thyroid lesions [39, 67, 77, 130, 146]. This problem is acknowledged in 2015 American Thyroid Association Management Guidelines for Adult Patients with Thyroid Nodules and Differentiated Thyroid Cancer (Haugen et al., Thyroid. 2015 Oct 14. [Epub ahead of print]) emphasizing the importance of standardization of molecular testing methodology.

Treatment

Currently, there is no effective therapy for advanced metastatic radioiodine-resistant thyroid cancer. Despite treatment with TSH suppression and local control with surgery and radiation therapy, the disease ultimately progresses causing significant mortality.

Greater understanding of thyroid cancer pathophysiology and the development of new therapies targeting specific molecular defects hold great promise for a pharmacologic cure of advanced thyroid cancer. Pharmacogenomics study how to use genetic and genomic information in clinical practice. This new field of medical science has emerged as a result of advances in molecular characterization of the disease and the development of new so-called targeted drugs aimed at a particular molecular defect. Pharmacogenomics is bringing medicine to a new level when not heterogeneous disease itself, but a molecular defect causing a particular instance of the disease, is targeted by a physician. For example, long-term control of chronic myeloid leukemia is achieved with inhibitors of the chimeric BCR-ABL oncoprotein (imatinib, dasatinib). BRAF inhibitors vemurafenib and dabrafenib produce rapid tumor regression in 80 % of patients with metastatic melanoma caused by *BRAF V600E* mutations. Figure 3.2 illustrates the power of pharmacogenomics. Cell lines from tumors of various origins, including thyroid cancer, were tested for sensitivity to the RAF inhibitor, PLX4720 [58]. Those cell lines that had the *BRAF V600E* mutation, but not the wild type gene, were selectively sensitive to the drug in an *in vitro* proliferation assay.

Unfortunately, the success of targeted therapies in thyroid cancer has been limited so far. In contrast to melanoma, only 25–35 % of patients with radioiodine-resistant metastatic thyroid cancer caused by the *BRAF V600E* mutation respond to BRAF inhibitors [45]. Currently, there are no effective therapies for tumors harboring oncogenic *RAS* mutants. A combination treatment with inhibitors of both the PI3K and MAPK pathway downstream of RAS has shown promise in animal models [6, 43], but this has yet to be tested in human thyroid cancer. The multikinase inhibitor, sorafenib, has been recently approved by the US Food and Drug Administration for the treatment of advanced radioiodine-resistant PTC based on the results of the DECISION trial [13]. Two targeted therapies, vandetanib and cabozantinib, are approved for advanced metastatic MTC. Both drugs are capable of inhibiting mutated RET as well as other targets [18, 151]. Vandetanib and cabozantinib were shown to prolong progression-free survival but not overall survival in phase 3 clinical trials in MTC [40, 144]. It remains controversial whether the presence of a *RET* mutation provided a therapeutic advantage in these trials. In patients who fail treatment with vandetanib or cabozantinib, the National Comprehensive Cancer Network recommends that clinicians consider additional targeted therapies with sorafenib or sunitinib or enrollment in a clinical trial [140]. Of note, sorafenib, sunitinib, and another tyrosine kinase inhibitor, ponatinib,

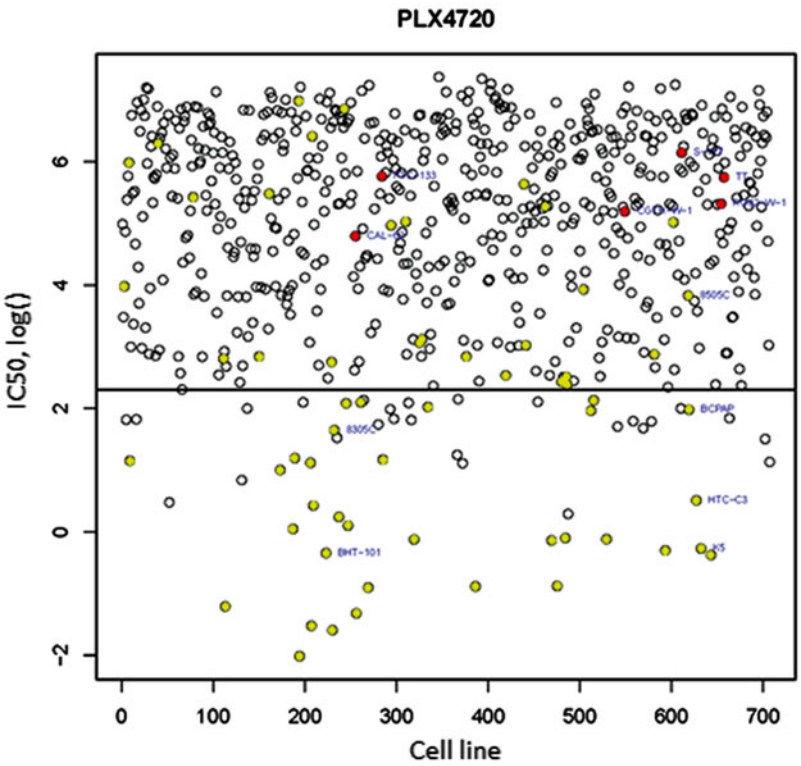

Fig. 3.2 Cell lines carrying the *BRAF V600E* mutation are sensitive to RAF inhibitor PLX4720. IC_{50} is a drug concentration that reduced the cell population by half in an *in vitro* proliferation assay. Thyroid cancer cell lines are named. *Yellow dots* represent cell lines with the *BRAF* mutation. *Red dots* represent thyroid cancer cell lines without the *BRAF V600E* mutation

are capable of blocking phosphorylation of the *RET V804M* mutant, which confers resistance to vandetanib and reduces the effectiveness of cabozantinib [17, 106]. Targeted therapies improve progression-free survival in clinical trials, but ultimately resistance develops and disease progresses in the majority of patients justifying the need for further efforts in developing pathogenesis-based therapies.

While infrequent, activating *ALK* gene mutations may have great therapeutic implications. A dramatic response to an ALK inhibitor, crizotinib, has been reported in a case of ATC with the *ALK* gene rearrangement [60]. Stable disease was achieved with crizotinib treatment in a patient with advanced PTC due to an *EML4-ALK* gene fusion [31]. The efficiency of ALK inhibitors in this subset of thyroid cancer has yet to be tested in a clinical trial.

After this chapter had been submitted for publication, The Cancer Genome Atlas Project dedicated to PTC was completed [15]. Multiplatform analysis of 496 PTC identified putative oncogenic drivers in 98.8 % of cases. New mutations and fusions were found. Higher somatic mutation frequency, the presence of TERT promoter mutations, and BRAF-like molecular signature were associated with advanced age, greater risk of recurrence, and higher MACIS prognostic score. Follicular variant of PTC was found to be distinct on a molecular level, and its reclassification into a follicular thyroid carcinoma group has been suggested.

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