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Coding molecular determinants of thyroid cancer development and progression

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Abstract

Thyroid cancer is the most common endocrine malignancy and its trends of incidence are the highest compared to other tumors. The characterization of the molecular pathways involved in thyroid cancer initiation and progression has made huge progress, underlining the essential role of determined signaling to promote specific features, including dedifferentiation, invasiveness, metastasis and drug resistance. The discovery of many genetic alterations that include point mutations, chromosome translocations, deletions, and copy-number gain has provided new exciting biological insights with translational/clinical applications. Furthermore, mechanisms of drug resistance involve role of pericytes and deregulation of pro-angiogenic molecules such as the tyrosine kinases (TKs) in the microenvironment. BRAF^{V600E}-positive thyroid tumor growth is also influenced by the tumor microenvironment, which is in turn altered by the tumor itself, leading to abnormal extracellular matrix (ECM) deposition and activation of angiogenic pathways. Many of the processes involved in thyroid tumor growth and metastasis are mediated by signaling molecules downstream of BRAF^{V600E} and activated TKs. Overall, understanding how molecular pathways interplay is one of the key-strategies to develop new therapeutic treatments and improve prognosis.

Keywords

BRAF^{V600E}; thyroid carcinoma; hTERT; PAX8/PPRγ; CDKN2A; RAS; microenvironment

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Introduction

Thyroid cancer is not only the most common malignancy of the endocrine system, but also has the highest rate of incidence amongst all tumor types in the United States¹, overall increasing 3% annually². The incidence-based mortality is typically low, however from 1994 to 2013 an increase of approximately 1.1% per year² has been observed.

According to the cellular origin, thyroid cancer is classified in two main histological types. Parafollicular or C cells have a neuroendocrine function, the production and secretion of calcitonin hormone, and originate medullary thyroid cancer (MTC), a small fraction of all malignancies (3–5%) while the majority of malignancies arise from follicular cells, responsible for thyroid hormone synthesis. Follicular cell-derived tumors are subsequently divided in subtypes according to their differentiation: differentiated thyroid cancer (DTC) that include papillary thyroid cancer (PTC) (80–85%) and follicular thyroid cancer (FTC) (10–15%); poorly differentiated thyroid cancer (PDTC); and anaplastic thyroid cancer (ATC) (collectively 1–2%)³. Conventional treatment for thyroid malignancy is characterized by surgical thyroidectomy followed by adjuvant radioiodine ablation in case of radioiodine uptake in the tumor foci⁴. Unfortunately, recurrences are not unfrequent, with high risk of long distance metastasis⁴. In the past ten years, the understanding of the molecular mechanisms that occur to thyroid cancer pathogenesis has greatly increased which has led to the continuous discovery of new therapeutic strategies. This article addresses the important developments towards greater knowledge of genetic alterations of molecular determinants with coding capabilities (oncogenic proteins, mutant tumor suppressors) in follicular-derived thyroid cancers.

Genetic alterations in early thyroid tumorigenesis

Gene mutations

BRAF—BRAF belongs to a family of serine/threonine kinases and works as downstream effector of RAS. BRAF transmits the signal through the mitogen-activated protein kinase (MAPK) pathway that has a fundamental role in promoting cell proliferation and survival⁵. In physiological conditions, MAPK signaling has a negative feedback mechanism mediated by ERK1/2, the principal downstream effector⁶. In 2002, the T1799A point mutation located in the exon 15 *BRAF* gene was first described in several human malignancies⁷. In presence of this missense nucleotide substitution, the residue 600 switches from glutamic acid to valine and causes the constitutive serine/threonine kinase activity with loss of inhibition loop. BRAF^{V600E} is the most frequent genetic alteration in melanoma⁸, hairy cell leukemia⁹ and PTC^{10,11}. PDTC and ATC also harbor BRAF^{V600E} with high prevalence (~33% and ~45%, respectively)¹² but the mutation is absent in FTC¹³. A less prevalent BRAF point mutation has been described in K601E residue in follicular thyroid adenoma (FTA)¹⁴ and follicular variant of PTC^{15,16}. Generally, tumors harboring this mutation show a follicular pattern and have a better clinical outcome¹⁶.

The presence of BRAF^{V600E} in micro PTC suggests its role as driver in thyroid tumor initiation¹³. In-fact, BRAF^{V600E} conditional expression is able to induce dedifferentiation and genomic instability in rat normal thyroid cells¹⁷. Moreover, BRAF^{V600E} detection in

PTC has been significantly associated with increased mortality¹⁸ and poorer clinic-pathological outcomes including increased aggressiveness, risk of recurrence, loss of radioiodine avidity and eventually therapy failure¹⁹. Transgenic mice carrying BRAF^{V600E} mutation and xenograft tumor models confirmed its essential role in the carcinogenic process and development of aggressive features^{20,21}. Given the high relevance of this genetic alteration, the clonality of BRAF^{V600E} has been debated, since studies were reporting discordant results^{22,23}. A more conclusive evidence from TCGA genome sequencing has provided that BRAF^{V600E} is a driving mutation clonally present in the PTC cells^{10,23}.

Importantly, BRAF^{V600E} is also deeply involved in the modulation of microenvironment to promote tumor progression and aggressiveness²⁴. BRAF^{V600E} modifies immune cells infiltration in PTC, and correlated with increased levels of some chemokines^{25,26}. Furthermore, BRAF^{V600E} PTC more often express high levels of immunosuppressive ligands programmed death ligand 1 (PD-L1) (53% vs. 12.5%) and human leukocyte antigen G (41% vs. 12.5%) compared to BRAF wild-type tumors²⁷. Importantly, RNAseq analysis has linked BRAF^{V600E} with overall decreased expression of immune and inflammatory response genes compared with BRAF^{WT}, leading to the hypothesis of a general immune escape mechanism operated by this oncogene²⁸. Moreover, the overexpression of vascular endothelial growth factor A (VEGFA)²⁹, metalloproteinase (MMPs)³⁰, fibronectin and vimentin³¹, transforming growth factor beta (TGFβ)^{24,32,33}, Thrombospondin 1 (TSP-1)²⁴ and p21-activated kinase (PAK)³⁴ have been correlated to BRAF^{V600E}. It is clear that the interplay between thyroid tumor cells and microenvironment is crucial to determine tumor aggressiveness and progression. Tumor cells and microenvironment stromal cells can interact through paracrine and autocrine signaling loops stimulating tumor progression. A recent study showed that tumor microenvironment pericytes play an essential role in protecting human thyroid cancer cells against therapy targeting BRAF^{V600E} (i.e. vemurafenib) and tyrosine kinases (TKs) (i.e. sorafenib) *via* TSP-1/TGFβ1 axis³⁵ (Figure 1). Specifically, pericytes were able to secrete both TSP-1 and TGFβ1, triggering drug resistance. BRAF^{WT/V600E}-PTC clinical samples were enriched in pericytes, and TSP-1 and TGFβ1 expression evoked gene-regulatory networks and pathways in the microenvironment essential for BRAF^{V600E}-PTC cell survival. Critically, antagonism of the TSP-1/TGFβ1 axis reduced tumor cell growth and overcomes drug resistance³⁵. Antagonizing this molecular pathway may represent a novel therapeutic translational approach against BRAF^{WT/V600E}-PTC resistant to targeted therapies.

BRAF^{V600E} could also be responsible for some clinical symptoms: in ATC the production and secretion of different angiogenic and pro-inflammatory key-factors (e.g. VEGFA, VEGFC, IL6) may contribute to the worsening of pathological features such as cachexia (the result of complex metabolic alterations which cause morbidity)³⁶. Overall, these results highlight the complexity of tumor interaction with the microenvironment, and how this interplay can affect thyroid cancer progression and consequently patient prognosis.

RAS—RAS is a G protein or guanosine-nucleotide-binding protein; specifically, RAS proteins belong to the small GTPases family and they are upstream to several intracellular signaling pathways³⁷. The GTP bounding promotes their activation; RAS proteins have an intrinsic hydrolysis activity that hydrolyzes a bound GTP molecule into GDP, which brings

it back to the inactive state³⁸. RAS mutations occur in thyroid carcinomas^{39–41}. Point mutations in 3 different codons (12, 13 and 61) are associated constitutive aberrant activation of downstream effectors⁴². This protein family is composed of three genes: HRAS, KRAS and NRAS. All three mutant RAS are associated with cancer including thyroid cancer^{39,43–45}. Both MAPK and PI3K (phosphatidylinositol-3-kinase)-AKT pathways are regulated by RAS, and thyroid tumors with RAS mutations also show over-activation of PI3K-AKT^{46,47}. Intriguingly, RAS mutations are more frequent in follicular variant of PTC or FTC than classical PTC^{10,44,47–51}. Follicular variant of PTC can harbor RAS mutations⁵². However, noninvasive follicular thyroid neoplasm with papillary-like nuclear features⁵³ (NIFTP), which have a very low risk of adverse outcome, also showed RAS mutations⁵³. RAS oncogene activation might play a role in early events of thyroid tumorigenesis, inducing proliferation in normal human thyroid epithelial cells without loss of differentiation cells^{54,55}. Interestingly, studies from a transgenic mouse model confirmed that mutant KRAS requires additional genetic alterations (such as PTEN deletion) to develop thyroid cancer, leading to invasive and metastatic FTC⁵⁶.

Gene translocations

RET/PTC—The most common gene translocation in PTC is RET/PTC. RET gene is encoded for a trans-membrane tyrosine kinase (TK) receptor and in condition of spatial contiguity with another gene⁵⁷ the 3' portion can be fused with more than 10 other genes at their 5'. The most frequent rearrangements are RET/PTC1 and RET/PTC3^{58,59}, respectively nuclear receptor co-activator 4 (NCOA4 also known ELE1 or RFG) and coiled-coil domain-containing gene 6 (CCDC6 also known as H4)^{60,59}. In the presence of RET/PTC translocation the fused protein keeps the kinase domains in the C-terminal and acquires a new N-terminal that provides to ligand-independent dimerization and constitutive TK activity. The pathogenic cause to this translocation event has been associated to the co-localization of chromosomal fragile sites, genome regions prone to DNA breakage⁶¹. Variability in RET/PTC prevalence is due to clonal or sub-clonal presence in the thyroid tumor. Clonal rearrangements are specific for PTC and occur in 10–20% of cases⁶² while the presence of RET/PTC in a small fraction of tumor mass has been detected in other thyroid cancer variants and also in benign thyroid lesions^{63–66}. Interestingly, RET fusions have a small prevalence in PDPTC (6%) but are absent in ATC, not showing overlap with point mutations¹².

PAX8/PPAR γ —Another relevant gene rearrangement (somatic translocation) in thyroid cancer is the fusion between the paired box 8 (PAX8) and proliferator activated receptor gamma (PPAR γ) (PAX8/PPAR γ). PAX8 is an essential transcription factor for thyroid gland development^{67,68} and in the mature organ it is responsible for thyroid-specific gene expression⁶⁸. Instead PPAR γ , a nuclear hormone receptor, has no known physiological role in the thyroid, but is well described as promoter of anti-inflammatory phenotype in macrophage⁶⁹ and as master regulator of adipogenesis⁷⁰. First described in 2000⁷¹ the PAX8-PPAR γ translocation has been detected with high prevalence in FTC (~50%), in a small fraction of follicular variant of PTC (1–5%) and also in FTA (2–13%)^{72,73}. PAX8/PPAR γ rarely overlaps with RAS point mutation, another common alteration of FTC, probably indicating distinct pathogenic pathways involved in tumorigenesis³⁹. In PTCs, the

detection of PAX8/PPAR γ is strongly associated with follicular features: tumors are encapsulated and likely have an indolent clinical course⁷⁴. PAX8/PPAR γ has intact DNA binding domain (DBD) and it is able to target DNA binding sites of transcriptional factors with a pro-adipogenic expression profile, preferentially activated in thyroid cells with this rearrangement⁷⁵. In thyroid cells, PAX8/PPAR γ expression can induce activation of the WNT/TCF pathway, causing an increase of invasiveness and aggressiveness features, like anchorage-independent growth⁷⁶.

A transgenic mouse model showed that PAX8/PPAR γ (PPFP) itself was not sufficient to induce tumor initiation without other mutation such as PTEN deletion. Whereas mice with combined PPFP and PTEN deletion developed metastatic thyroid cancer, consistent with patient data that PPFP is occasionally found in benign thyroid adenomas and that PPFP carcinomas have instead increased phosphorylated AKT/protein kinase B⁷⁷. Chip-seq analysis on the mentioned mouse model (PPFPxPten⁻) showed enrichment in binding on genes involved in fatty acid metabolism, cell cycle regulation and WNT signaling⁷⁸. Furthermore, treatment with a PPAR γ agonist (pioglitazone) showed a significant increase of immune cells infiltration compared to the control, suggesting a potential therapeutic effect for this type of thyroid cancer⁷⁸ (Figure 2).

NTRK—The neurotrophic tyrosine kinase receptor (NTRK) family is involved in sporadic rearrangements in thyroid cancer. NTRK1 gene, which resides in chromosome 1q, can have different partners such as TPR^{79,80} TMP3^{81,82}, TFG⁸³. The fused protein has constitutive kinase activity, with promotion of downstream pathways. The prevalence of this genetic alteration is still debated, with previous studies showing about 11.8% in PTC⁸⁴ and more recent analysis decreasing up to 1–2%⁸⁵, even there is accordance in an higher frequency among young patients. NTRK3/ETV6 fusions was exclusively found in 13% of follicular variant of PTC, in both encapsulated and infiltrative variants, but was not found in FTAs and FTCs⁸⁶. NTRK3/RBPMS is another fusion that occurs in 1.2% of PTC¹⁰. Importantly, ETV6/NTRK3 fusion is more common (14.5%) in radiation-exposed population (post-Chernobyl PTCs) and in 2% sporadic PTCs⁸⁷.

STRN/ALK—RNAseq analysis has recently allowed the identification of a novel gene fusion in thyroid cancer, involving the anaplastic lymphoma kinase (ALK) gene and the striatin (STRN) gene⁸⁸. This event is the result of a complex rearrangement involving the short arm of chromosome 2. The fusion between exon 3 of STRN and exon 20 of ALK leads to protein dimerization (mediated by the coiled-coil domain) of STRN and subsequent ALK constitutive kinase activity. In thyroid cancer cells STRN/ALK rearrangement stimulates proliferation independently from TSH signaling, furthermore inducing tumor transformation⁸⁸. STRN/ALK has been detected in PTC^{86,88,89}, in follicular variant of PTC^{86,88}, in PDPTC and ATC⁸⁸. Interestingly, STRN/ALK fusion tumors are negative for other driver mutations, indicating its role in thyroid tumorigenesis and suggesting that it may represent a therapeutic target for patients with this type of tumors.

AKAP9/BRAF—A rare gene fusion in radiation-induced PTC via paracentric inversion of chromosome 7q resulting in an in-frame fusion between exons 1–8 of the AKAP9 gene and exons 9–18 of BRAF. The fusion protein contains the protein kinase domain and lacks the

autoinhibitory N-terminal portion of BRAF⁹⁰. The prevalence of this fusion protein in sporadic PTC is very low and can be considered a rare event^{91,92}. This molecular fusion has elevated kinase activity and transforms NIH3T3 cells.

Genetic alterations and pathways deregulation associated with thyroid cancer progression

Additional mutations that occur in thyroid cancer are very often associated with cancer progression and tumor aggressive features.

WNT/β-catenin signaling pathway—The WNT/β-catenin pathway has a key role in multiple cell processes, such as cell growth and proliferation, cell adhesion, and stem cell differentiation. If altered (e.g. mutation on WNT receptors), it can commonly lead to constitutive activation in human tumors⁹³. In thyroid cancer, this process is caused by mutations on the CTNNB1 gene (encoding for β catenin) that occurs with high prevalence in PDPTC and ATC (up to 60%)^{94–96}. The consequence is the impairment of physiological β-catenin degradation, thus allowing cytoplasmic accumulation and translocation into the nucleus and eventually promoting transcription of genes involved in tumorigenesis, for example cell cycle regulators^{97,98}. Additionally, no β-catenin mutations have been detected in DTCs, leading to the association of this pathway to increased aggressiveness. However, β-catenin nuclear aberrant translocation and localization can be caused by other mechanisms, including post-translational modifications⁹³. It is well known that PI3K/AKT pathway deactivates (by AKT direct phosphorylation) GSK3β, a key promoter of β-catenin ubiquitination and of further degradation⁹⁹. AKT phosphorylates β-catenin at serine (Ser, S) 552, which leads to its disassociation from cell-cell contacts, increases its binding to 14–3–3 and its transcriptional activity, and enhances invasion by tumor cells its activity¹⁰⁰. Furthermore, ERK1/2 can inhibit GSK3β resulting in up-regulation of β-catenin¹⁰¹. In thyroid cells, β-catenin is activated physiologically by PI3K signaling in response to TSH and IGF1¹⁰² but it has also been demonstrated that RET/PTC translocation and HRAS mutation (but not BRAF) activate β-catenin in cancer cells^{103–106}. This BRAF^{V600E} independency is still controversial, since up-regulation of β-catenin has been found in PTC and ATC cells with BRAF^{V600E}¹⁰⁷. However, another study based on immunohistochemical and microarray analysis showed differential β-catenin up-regulation in BRAF^{WT} vs. BRAF^{V600E} PTCs¹⁰⁸. Additional work is probably needed to understand what drives abnormal activation of WNT-β-catenin in a physiological context and which molecular partners are involved.

TP53—Mutations leading loss of function of the tumor suppressor and cell cycle regulator p53 were first described as a unique characteristic of thyroid cancer dedifferentiation with high prevalence (50–80%) in ATC^{109–111}. Further analysis with mouse model generation and targeted next-generation sequencing have confirmed the correlation between TP53 alterations and worse pathological features of ATC^{112,113}.

TERT—In 2013, two point mutations on TERT gene promoter were detected in thyroid carcinoma, C228T and C250T^{114,115}. The TERT gene encodes the reverse transcriptase subunit of the telomerase complex, the specialized DNA polymerase that elongates the telomere portion of chromosomes adding repeated sequences. Its expression and activity is

usually absent/low in normal cells whereas is strongly increased in cancer cells¹¹⁶, including aggressive thyroid cancers^{117,118}. The mutations in the promoter of TERT gene were found in different cancers^{119–121}, determining a consensus binding site for ETS transcription factors. C228T and C250T are both present with lower prevalence in PTCs (~10%) compared to PDTCs (40%) and ATCs (~70%)¹². A significant co-occurrence with mutations of BRAF and RAS suggested TERT role as an acquired genetic alteration that allows extended survival to clones with preexisting driver mutations and subsequently leads to cancer progression¹¹⁴. This hypothesis has been confirmed by different studies showing TERT promoter mutations: (i) with higher prevalence in FTCs and aggressive BRAF^{V600E}-positive PTCs¹²² and no TERT mutation was found in benign thyroid lesions; (ii) correlated with a worse clinical outcome in patients with DTCs¹²³; (iii) coexistence with BRAF^{V600E} has a robust synergistic impact on tumor aggressiveness of PTCs, including poor clinic-pathological characteristics^{122,124} and increased patient mortality¹²⁵; and (iv) significantly correlated with BRAF^{V600E}, older patient age, and tumor distant metastasis in ATC¹²⁶. Importantly, the molecular mechanism of synergistic coexistence between BRAF^{V600E} and TERT promoter mutations in cancer has been recently elucidated: BRAF^{V600E} through its effector ERK1/2 enhances MYC expression and FOS phosphorylation and the latter promotes GABPB expression. MYC and GABPB are both transcriptional factors that bind TERT promoter and trigger its overexpression, with the effect of GABPB being in a TERT mutation-dependent manner¹²⁷ (Figure 3).

EIF1AX—The eukaryotic translation initiation factor EIF1AX was first discovered mutated in uveal melanomas¹²⁸ and this genetic alteration has been reported as largely mutually exclusive with BRAF and RAS in 1% of PTCs¹⁰. The prevalence increases in PDTCs (11%) and in ATCs (9%) and EIF1AX is strongly correlated with RAS¹². The most prevalent mutational event consists in a splice mutation causing C-terminal 12-amino acid in-frame. EIF1AX mutations are also predictive of low survival in PDTC^{10,12}.

PI3K-AKT pathway mutations and deregulations—The PI3K pathway is composed of a large group of proteins that control several cellular processes including cell proliferation, survival and motility¹²⁹. In physiological conditions, PI3K is recruited in the internal layer of plasma membrane by protein adaptors that can be activated by multiple transmembrane tyrosine kinase receptors. Moreover, some isoforms have a binding domain that permits activation from RAS. PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) in phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 is a second messenger promoting the activation of effectors such as PDK1 and AKT. Genetic alterations carried by partners in PI3K-AKT pathway are strongly correlated to thyroid cancer progression. Overall, PI3K-AKT signaling shows an important role in thyroid oncogenesis¹³⁰.

PTEN, a protein tyrosine phosphatase with homology to tensin, is a tumor-suppressor gene that encodes a protein and lipid phosphatase. Specifically, PTEN inhibits PI3K-AKT pathway promoting PIP3 de-phosphorylation. The first evidence that linked PTEN loss of function mutations with thyroid cancer came from a study on Cowden's syndrome, a congenital disease characterized by germline mutations of PTEN, that shows a strong

predisposition FTA and FTC¹³¹. Subsequently, the importance of PTEN as a tumor suppressor has been demonstrated in heterozygous PTEN^{+/-} mouse model that spontaneously develop, amongst the others, thyroid and colon cancer¹³². However, the complete PTEN loss in the thyroid is not sufficient to originate invasive tumors as has been shown in a tissue-specific transgenic mouse model, suggesting the importance of additional genetic alterations in order to promote thyroid transformation¹³³. In human thyroid tumors the most common genetic alteration associated is somatic deletions of the *PTEN* gene (~5–25%) due to loss of heterozygosity (LOH) on chromosome 10^{134,135}. PTEN frameshift mutations can occur in ATC¹¹³; also, PTEN mutation are more frequent in ATC (15%) compared to PDTC¹².

Somatic mutations in exon 9 and exon 20 of PIK3CA, encoding the PI3K catalytic subunit p110a, are frequent in many human tumors¹³⁶, including DTC (~8%)¹³⁷ and ATC (~25%)^{138,139}, with an interesting co-occurrence with BRAF^{V600E} in the undifferentiated tumors¹². In presence of these genetic alterations, the kinase is not sensitive to its regulatory subunit, and is not deactivated¹⁴⁰. Mutations on other PI3K subunits have been observed in PDTCs and ATCs but with lower prevalence¹².

AKT is the downstream effector of PI3K signaling and is recruited by PIP3 and subsequently phosphorylated and activated by PDK1 and mTOR (mechanistic target of rapamycin). AKT is a serine/threonine kinase and has three different isoforms, encoded by different genes all regularly expressed in thyroid tissue that differ mainly for their regulatory and adaptor domains. In metastatic thyroid cancer AKT1 can be mutated¹⁴¹. mTOR is another serine/threonine kinase that exists in two complexes: mTORC1 (or mTOR-Raptor), activated by AKT, that promotes, among other functions, cell growth and biosynthesis of macromolecules, and mTORC2 (or mTOR-Rictor) that phosphorylates AKT (as mentioned above) and triggers cell survival processes¹⁴². MTOR gene mutation has been described in PDTC and ATC at low frequency (respectively 1% and 6%)¹². Overall it has been shown that over 50% of FTC and ATC harbored at least one PI3K-AKT pathway–related genetic alteration¹³⁷.

Additionally, PI3K-AKT signaling can be abnormally active in absence of mutation. AKT1 and AKT2 are overexpressed and over-activated particularly in FTC¹⁴³. AKT1 nuclear translocation has been also linked to capsular invasiveness, cell invasion and migration¹⁴⁴. AKT1 deletion in PTEN^{+/-} mouse model sufficiently inhibits tumor development¹⁴⁵ and AKT2 deficiency does not have the same ubiquitous effect as AKT in PTEN^{+/-} mice, but still shows a decreased incidence of thyroid tumors¹⁴⁶.

Gene copy number variations—Copy number variations (CNV) (including gene amplification) are additional mechanisms that critically contribute to carcinogenesis^{147,148}. They are due to chromosomes instability and aneuploidy that permits the acquisition of a genetic advantage promoting pathogenic signaling^{148,149}. Genes encoding for various types of tyrosine kinase (TKs) receptors, including EGFR, PDGFRA, PDGFRB, VEGFR1, VEGFR2, etc. have been detected in FTCs and ATCs, in association with increased activation of phosphorylated AKT and ERK1/2⁴⁷. Genes of the PI3K-AKT pathway that are also amplified include: PIK3CA, PIK3CB, AKT1 and AKT2^{47,137,139}. Interestingly,

PIK3CA mutation and PIK3CA amplification are mutually exclusive in DTCs^{137,150}, suggesting that one specific genetic alteration of PI3K-AKT pathway is sufficient for promoting thyroid tumorigenesis. Whereas accumulation of different genetic alterations occurs in ATC^{137,151}, increasing potential of metastasis and tumor progression.

In PTC copy number variations include loss on chromosome 22 (q arm)^{10,146} in a region that shows sequence of the NF2 and CHEK2 gene and reported to be lost with significant frequency; amplification on chromosome 1q; and gain of 5p and 5q often in association with BRAF^{V600E} mutation¹⁰. NF2 gene encodes for a tumor suppressor protein, which inhibits cell growth in response to cell-to-cell contact. NF2 loss augments mutant RAS signaling, strengthening MAPK signaling, in part through inactivation of Hippo, which activates a YAP-TEAD transcriptional program in murine model of PDTC¹⁵². Moreover, BRAF^{V600E} PTCs with metastasis showed amplification of 26 genes on chromosome 1q including MCL1 (anti-apoptotic gene belonging to BCL2 family) and P16 gene (CDKN2A) loss on chromosome 9p¹⁵³. P16 is a cell cycle negative regulator, impairing CDK4/6 complexes formation and Rb phosphorylation during G1 phase¹⁵⁴ (Figure 4). For this reason, CDK4/6 inhibitors in combination with BRAF^{V600E} inhibitor (vemurafenib) have been demonstrated to strongly induce apoptosis in PTC and ATC cells with the heterozygous BRAF^{V600E} mutation and with P16 loss¹⁵⁵. DNA CNVs might occur on Chromosome 7, 12 and 17 in thyroid lesions¹⁵⁶. Overall, CNVs frequency is higher in aggressive thyroid tumors and ATC, indicating a role in tumor progression.

NF-κB signaling pathway—NF-κB (Nuclear factor-kappa B) signaling plays an important role in cancer development and progression, providing a mechanistic link between inflammation and cancer¹⁵⁷. NF-κB is able to induce tumor proliferation, block apoptosis, and promote angiogenesis and invasion¹⁵⁷. From many years, it has been known that in thyroid cancer cells NF-κB pathway has a pro-oncogenic role^{158–160}. More specifically, it has been demonstrated that the genetic alterations mainly occurring in thyroid carcinogenesis are converging in activation of NF-κB are: BRAF^{V600E} in a MEK-independent manner¹⁶¹; RET/PTC3, through stabilization of NF-κB-inducing kinase (NIK)¹⁶²; PAX8/PPARγ, due to reduced PPARγ protein abundance¹⁶³. Moreover, constitutive activation of the PI3K-AKT pathway due to PTEN inactivation increases NF-κB activity, thus accelerating thyroid cancer progression¹⁶⁴. NF-κB role in angiogenesis and metastasis promotion has been further characterized in a mouse model of ATC and PDTC harboring BRAF^{V600E}, providing an association with IL-8 secretion¹⁶⁵. However, NF-κB inhibitors administered in combination with classical chemotherapy and radiation are not sufficient to target thyroid cancer cells¹⁶⁶, suggesting that further investigations are needed to understand which subset of patients/tumors could respond to this therapeutic target.

RCAN1–4: RCAN1 (Regulator of calcineurin 1, also known as Down syndrome candidate region 1) is a gene located in the chromosome 21 with multiple transcriptional start sites that expresses two main isoforms, RCAN1–1 and RCAN1–4¹⁶⁷. RCAN1–4 is a competitive inhibitor for the phosphatase calcineurin and thereby suppresses calcineurin-mediated dephosphorylation and activation of nuclear factor of activated T cells (NFAT)¹⁶⁸. Since

NFAT is a transcriptional factor promoting RCAN1–4 gene expression, RCAN1–4 can be considered a mediator of a negative feedback loop on this pathway¹⁶⁹.

Interestingly, RCAN1 has been correlated with tumor growth protection in individuals with Down syndrome¹⁷⁰, and its tumor suppressor activity is probably exerted by blocking the angiogenesis process (impairing endothelial cell migration, neovascularization, and tumor growth) through NFAT inhibition^{171–173}.

RCAN1 expression is increased in primary tumors versus normal tissue, but the expression is lost in metastases, a pattern consistent with a metastasis suppressor¹⁷⁴. Moreover, in different cancer cell lines (including FTC cells) RCAN1 reduces migration and alters cell adhesion¹⁷⁵. Recently, it has been reported that stable down-regulation of RCAN1–4 in human thyroid cancer cell lines increased cell viability and invasion *in vitro* and promoted tumor growth and metastasis in mouse xenograft models¹⁷⁶. These phenotypes were dependent on NFE2L3 (nuclear factor, erythroid 2-like 3), a transcription factor overexpressed in human thyroid cancer samples¹⁷⁶.

BRAF, RAS, RET/PTC overlapping and signaling cooperation between MAPK and PI3K-AKT—We already have discussed how progressive mutations on PI3K-AKT pathway accelerate and lead to thyroid tumor progression. In DTCs, genetic alterations on main oncogenic drivers such as BRAF, RAS and RET/PTC are mutually exclusive^{10,14,177}. Instead, in aggressive thyroid tumors, a potential overlap is still debated: BRAF^{V600E} mutation and RET/PTC translocations co-occurred in recurrent PTC¹⁷⁸, next-generation sequencing on PDTC and ATC showed mutual exclusivity for BRAF^{V600E}, RAS mutations, and gene fusions rearrangements¹². Intriguingly, driver mutations that potentially activate both MAPK and PI3K were reported^{139,141,151}, suggesting a precise time course of thyroid tumor initiation and progression. Therefore, the most common genetic alterations are mainly responsible for initial tumor transformation and lead tumor cells to a “cancer-prone environment” more susceptible to additional mutations (secondary or passenger mutations). With the progressive accumulation of deregulated signaling pathways and aberrant functional proteins (not only MAPK and PI3K-AKT, but also β -catenin, hTERT, TP53, etc.) the aggressiveness features (angiogenesis, cell adhesion, migration, invasion, and metastasis) worsens towards undifferentiated/anaplastic thyroid tumors which are well-known for having the highest mortality rates¹⁷⁹.

Thyroid hormone receptor β : a key-player in robust preclinical models of invasive FTC

Thyroid hormone receptors are ligand-dependent transcription factors that regulate cell growth, development and differentiation in response to thyroid hormone (T3). This protein family is entirely encoded from two genes, TR α and TR β . In 2000 a mouse model carrying a targeted mutation on TR β (TR β PV) was established to understand the molecular basis of resistance to thyroid hormone (RTH) syndrome, characterized by reduced sensitivity of tissues to the action of thyroid hormone¹⁸⁰. Those mice exhibited impaired growth and resistance to thyroid hormone. A couple of years later it was reported that mice homozygotes for PV mutation (TR β ^{PV/PV}) spontaneously develop metastatic FTC, showing aggressive features as anaplasia and metastasis¹⁸¹. Many studies have later focused on

elucidating the pathways contributing to tumor initiation in TR $\beta^{PV/PV}$ mice. PPAR γ mRNA expression is downregulated¹⁸² due to transcription repressive activity of TR β^{PV} on the peroxisome proliferator response element (PPRE)¹⁸³. More importantly, TR β^{PV} has been linked to PI3K-AKT pathway activation via interaction with p85 α (regulatory subunit of PI3K), well known for being a key regulator in thyroid tumorigenesis¹⁸⁴. Cancer progression and occurrence of metastasis is increased if combined with PTEN deficiency, causing AKT overexpression and over-activation, with significant effects on AKT downstream targets: increased mTOR-p70S6K signaling and inhibition of FOXO3a, a member of the forkhead family of transcription factor FOXO, promoter of pro-apoptotic factors transcription, and repressor of cyclin D1¹⁶⁴. Additionally, it has been demonstrated that T3 has a role in β -catenin suppression. T3 binding on TR β induces dissociation and subsequent degradation of β -catenin in cells via proteosomal pathways¹⁸⁵, and direct repression of CTNNB1 gene through interaction with negative thyroid hormone response elements (TREs) in the promoter¹⁸⁶. TR β^{PV} is refractory to this mechanism, stabilizing β -catenin and promoting oncogenesis. TR $\beta^{PV/PV}$ mouse model has further been developed following some strategy: high Fat Diet in TR $\beta^{PV/PV}$ Pten^{+/-} showed an increased level of circulating leptin with activation of JAK2-STAT3 pathway with higher occurrence of metastasis¹⁸⁷. STAT3 is a transcription factor promoter of cyclin D1, Myc and Bcl2 and crucial for metastasis; importantly, the leptin-JAK-STAT3 signaling role was confirmed using a STAT3 selective inhibitor¹⁸⁸ that reduced vascular invasion, anaplasia, and metastasis. Moreover, metformin treatments in high fat diet- TR $\beta^{PV/PV}$ Pten^{+/-} reduced capsular invasion and blocked anaplasia and vascular invasion but not thyroid tumor growth¹⁸⁹. The genetically targeted KRAS (G12D) mutation in thyroid epithelial cells of TR $\beta^{PV/PV}$ mice develop a more aggressive tumor, with frequent anaplastic foci showing lack of PAX8 expression, MYC up-regulation. Thus, MYC might serve as a potential target for therapeutic intervention and suggesting a signaling cross talk between RAS, PI3K/AKT and β -catenin pathways¹⁹⁰. A MYC inhibitor (JQ1) showed successful inhibition of tumor growth in TR $\beta^{PV/PV}$ -KRAS^{G12D} mice¹⁹¹. Also, combined treatment with JQ1 and metformin has been recently performed on high fat diet-TR $\beta^{PV/PV}$ Pten^{+/-} mice, reducing tumor growth by attenuating STAT3, decreasing anti-apoptotic key regulators and suppressing vascular invasion, anaplasia, and lung metastasis¹⁹².

Summary

In these past years, there has been remarkable progress in the understanding of the molecular signature (molecular determinants with coding capabilities) of thyroid cancer. Novel genetic alterations associated with clinic-pathological features of thyroid carcinomas have been discovered. The characterization of these genetic alterations revealed the essential role of MAPK and PI3K/AKT signaling in promoting thyroid tumor initiation and progression. These results have greatly led to the development of targeted therapies for precision medicine, inhibiting key-players of these pathways. Unfortunately, selective drug resistance has been constantly reported, explaining the need to identify new potential therapeutic targets in order to overcome tumor resistance. The interplay between different pathways in advanced thyroid cancers refractory to standard therapies (including radio-iodine) has been

recently better characterized, opening new possibilities of early treatment and improved prognosis.

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Key Points

- BRAF^{V600E}, RAS, RET/PTC, and PAX8/PPAR γ are the most characterized genetic alterations responsible for thyroid tumorigenesis, causing deregulation of MAPK (MEK1/2 and ERK1/2) and PI3K-AKT intracellular signaling.
- In the past years, other genetic alterations have been reported, including hTERT mutations.
- Accumulation of multiple mutations involving different pathways can lead to thyroid cancer progression.
- Whole genome sequencing has shed light on unknown genetic landscape, unraveling novel genomic alterations.

Tumor Microenvironment

Figure 1.
Role of pericytes in tumor microenvironment in promoting thyroid cancer cell survival via the TSP1/TGFβ1 axis.

Figure 2.

Transgenic mouse model with thyroid specific PAX8/PPAR γ translocation (PPFP) and PTEN deletion develops follicular thyroid carcinoma. At a molecular level, PPFP weakly induces a subset of adipocyte PPAR γ target genes, promotes cell cycle progression and activates the WNT/TCF pathway. Pioglitazone (a PPAR γ agonist that also binds PPFP) causes PPFP to strongly activate adipocyte PPAR γ target genes, resulting in trans-differentiation of the thyroid cancer cells into adipocyte-like cells. In addition, pioglitazone-activated PPFP recruits immune cells in the tumor microenvironment.

Figure 3.

Human (h) TERT promoter mutations and BRAF^{V600E} elicit synergistic pathways in thyroid carcinoma cells. BRAF^{V600E} *via* ERK1/2 phosphorylation promotes MYC expression and FOS phosphorylation. FOS is a transcription factor that regulates GABPB expression. MYC and GABPB are both transcriptional factors responsive to TERT promoter mutations and enhance mutant hTERT expression.

Figure 4. BRAF^{V600E} and P16 loss cooperate to promote thyroid tumor survival through phosphorylation of ERK1/2 and AKT, activating CDK4/6 and inactivating Rb by phosphorylation.