ENDOCRINE TUMOURS

Advances in the molecular pathogenesis of thyroid cancer: lessons from the cancer genome

Garcilaso Riesco-Eizaguirre¹,² and Pilar Santisteban¹

¹Instituto de Investigaciones Biomédicas “Alberto Sols” Consejo Superior de Investigaciones Científicas and Universidad Autónoma de Madrid (CSIC-UAM), Madrid, Spain and ²Servicio de Endocrinología, Hospital Universitario de Móstoles, Madrid, Spain

Abstract

Thyroid cancer is the most common endocrine malignancy giving rise to one of the most indolent solid cancers, but also one of the most lethal. In recent years, systematic studies of the cancer genome, most importantly those derived from The Cancer Genome Altas (TCGA), have catalogued aberrations in the DNA, chromatin, and RNA of the genomes of thousands of tumors relative to matched normal cellular genomes and have analyzed their epigenetic and protein consequences. Cancer genomics is therefore providing new information on cancer development and behavior, as well as new insights into genetic alterations and molecular pathways. From this genomic perspective, we will review the main advances concerning some essential aspects of the molecular pathogenesis of thyroid cancer such as mutational mechanisms, new cancer genes implicated in tumor initiation and progression, the role of non-coding RNA, and the advent of new susceptibility genes in thyroid cancer predisposition. This look across these genomic and cellular alterations results in the reshaping of the multistep development of thyroid tumors and offers new tools and opportunities for further research and clinical development of novel treatment strategies.

Introduction

Thyroid cancer is the most prevalent type of endocrine malignancy, and its incidence has been steadily increasing over the last three decades (1). Due to this rise in its incidence, thyroid cancer is currently the fifth most common new cancer diagnosis in women and the eighth most common new cancer diagnosis overall in the USA (2). It is now more frequently diagnosed than all leukemias combined, as well as ovarian, uterine,

Invited author’s profile

Pilar Santisteban has a PhD in Biology from Autonomous University of Madrid. Currently she is a Research Professor at the Spanish National Council of Research (CSIC), in the Biomedical Research Institute “Alberto Sols” of Madrid, where she is directing the group of “Molecular and Cell Biology of the Thyroid”. She has conducted various training periods in recognized international centers studying several approaches of thyroid pathophysiology. Her current work is dedicated to the study of emerging genes and pathways in thyroid development and cancer.

Garcilaso Riesco-Eizaguirre MD, PhD is a medical endocrinologist at the Móstoles Univesrity Hospital and collaborator at the Biomedical Research Institute “Alberto Sols” in Madrid (Spain). His research focuses on the understanding of the pathogenesis and molecular biology of thyroid cancer with the goal of identifying new molecular-based therapies.
pancreatic, or esophageal cancers. For the majority of patients with thyroid cancer, treatment with surgery, radioactive iodine (RAI) ablation, and TSH suppressive therapy allows an overall survival (OS) rate of 97.7% at 5 years (3). Nevertheless, locoregional recurrence occurs in up to 20% of patients and distant metastases in approximately 10% at 10 years. Some of these patients with locoregional recurrences and/or distant metastases lose the ability for iodine uptake leading to RAI-refractory metastatic disease. Patients with RAI-refractory metastatic disease have an overall survival rate of less than 50% at 3 years and account for more deaths in the USA at present than Hodgkin’s lymphoma, osteosarcoma, or testicular cancer.

From a clinical point of view, current management of thyroid cancer has suffered some important changes, but these changes are only beginning to be related with our increased understanding of its molecular pathogenesis. In order to prevent overtreatment, clinicians are making a big effort to better stratify thyroid cancer patients according to their risk of recurrence. Indeed, overtreatment has been a rule for the past 30 years, since Mazzaferri reported in the 80s the benefits of total thyroidectomy, radioiodide treatment and thyroid hormone suppression (4). Because this strategy was in general terms well tolerated and the survival rate was very high compared with other forms of cancers at that time, endocrinologists felt comfortable for a long while. Recent prospective and retrospective clinical studies are showing us that many patients would not benefit from this “one for all” strategy and recent thyroid cancer guidelines recommend stratifying the risk of the patients to determine different therapeutic strategies (5). For the first time, some molecular markers (i.e. BRAF and TERT) were recommended for such risk stratification along with clinical and pathological data. However, surely in the near future, the understanding of the molecular pathogenesis of thyroid cancer will provide new molecular markers and tools.

Based on hypothesis-driven research, the classic view of thyroid cancer pathogenesis considers thyroid carcinomas as tumors accumulating mutations that drive progression through a dedifferentiation process initially giving rise to well-differentiated carcinomas, such as papillary (PTC) and follicular (FTC), and progressing to poorly differentiated (PDTC) and undifferentiated or anaplastic (ATC) thyroid carcinomas (6, 7). Initiation and progression of thyroid cancer comprises multiple genetic alterations, of which mutations leading to the activation of the MAPK and PI3K/AKT signaling pathways are the most studied. MAPK activation is crucial for PTC initiation. The altered genes that affect this pathway include mutations in the intracellular signal transducers RAS and BRAF and rearrangements in the cell membrane receptor tyrosine kinases RET (RET/PTC), being these mutations mutually exclusive (8, 9). PI3K/AKT activation is thought to be critical in FTC initiation through mutations in RAS, inactivating mutations in the tumor suppression gene PTEN, or by activating mutations in the PIK3CA and AKT1. Some patients with PTC and FTC may progress to RAI-refractory metastatic disease, and these tumors are particularly enriched with mutations in BRAF and RAS coexisting with PIK3CA or AKT1 (10, 11). Thyroid cancer progression and dedifferentiation to PDTC and ATC involves a number of additional mutations that affect other cell signaling pathways such as p53 and Wnt/β-catenin (Fig. 1A).

In the past few years, with the availability of the genome sequence, comprehensive efforts have revealed the genomic landscapes of common forms of human cancer including thyroid cancer (12). This new field of cancer genomics is completing the mutational catalog in primary tumors and across the natural history of cancer, allowing us to connect genomic alterations to altered pathways and biological capabilities acquired during the multistep development of human tumors. In thyroid cancer, recent evidence provided by cancer genomics is starting to complete the picture resulting in the reshaping of the multistep model of thyroid cancer development (Fig. 1B). In this new genomic era, this review will discuss how these findings are helping us to better understand the essential aspects of thyroid cancer pathogenesis such as mutational mechanisms, new cancer genes implicated in tumor initiation and progression, the role of non-coding RNA, and the advent of new susceptibility genes in thyroid cancer predisposition.

**Mutational mechanisms**

**Mutation rates and mutation spectra**

The rate of genetic mutation vary substantially across the cancer genomes, ranging from as low as one base substitution per exome (0.1/Mb) in some pediatric cancers and leukemias to thousands of mutations per exome (100/Mb) in certain mutagen-induced malignancies such as melanoma and lung (Fig. 2) (13). These higher mutation rates reflect the involvement of potent mutagens such as ultraviolet light and cigarette smoke in the pathogenesis of melanoma and lung cancer respectively. In PTC, whole-exome sequencing showed a low mutation rate
Figure 1
Stepwise model of thyroid carcinogenesis. Panel A: Stepwise model of thyroid cancer adapted from Haugen and Sherman (Endocrine Reviews, 2013; 34: 439–455). This classic model is one of the most widely accepted for thyroid cancer progression in which the main genes (driver or not) involved in each step are indicated. The well-differentiated thyroid tumors toxic adenomas (TA), follicular adenomas (FA), follicular carcinomas (FTC), and papillary carcinomas (PTC) express thyroglobulin (TG) as well as a functional iodide symporter (NIS). The poorly differentiated tumors (advanced thyroid cancer refractory to radioiodide (RAIR)) express TG, but NIS is not expressed or is not functional. Finally, the undifferentiated tumors (anaplastic one (ATC)) does not express TG nor NIS. Panel B: The current knowledge based on the thyroid cancer genome has changed our vision of the classic model (TGCA 2014 (14) and Landa et al. 2016 (16)). The main oncogenic drivers associated with well-differentiated thyroid cancer (PTC and FTC) can be grouped together in two types: BRAF-like and RAS-like, according to their consequences in signaling and differentiation (BRS scoring system based on a 71 gene signature, see text). The follicular variant of the PTC is included in the RAS-like, consequently closer to FTC. Additional drivers such as mutations in TERT promoter, DNA repair genes, or gain of 1q are associated with progression to PTC/FTC RAIR. Progression to PDTC and ATC is determined by the initial drivers BRAF and RAS. RAS-mutated PDTC are enriched with loss of 22q and, both RAS-mutated PDTC and ATC are enriched specifically with mutations in EIF1AX and do not overlap with TERT, while BRAF-mutated ATCs are enriched with mutations in PI3K. Mutations in p53 are highly prevalent in all ATCs (73%) but much less in PDTC (8%). Mutations in components of the SWI/SNF chromatin remodeling complex and in the histone methyltransferases (HMTs) are new genes found to be associated with ATCs (36% and 24% respectively). Both RAS- and BRAF-mutated ATCs presumably derive from well-differentiated carcinomas (secondary ATCs). However, there are some ATCs that are BRAF and RAS wild type (wt), suggesting that a subset of these tumors could be considered as primary ATC (Kunstman, 2015 (15)). NF1 mutations are found in BRAF and RAS wt, in association with PTEN.
Thyroid cancer genome

G Riesco-Eizaguirre and P Santisteban

(11 non-synonymous mutation per tumor, 0.41 mutation per Mb on average), one of the lowest among solid tumors as the Cancer Genome Atlas of the Thyroid (TCGA) has demonstrated (14). This finding may explain the indolent clinical behavior of PTC, yet leukemias and pediatric cancers have also low mutation rates and are far from being indolent. Mutation rates are governed by processes such as replication timing and transcription-coupled repair. Thyroid cells have low replication timing and DNA repair genes are affected with low frequency in PTC. In contrast to PTC, ATC showed a much higher mutation rate (90 non-synonymous mutation per tumor, around 3 per Mb) (15). Mutations in DNA repair genes such as p53 are particularly prevalent in ATC and have been shown to play a causal role in genomic instability (16, 17). However, potent mutagens underlying such high mutation rate in ATC remain unknown.

Mutation rates also vary across patients within a cancer type (18). For example, in melanoma, the frequency ranges across 0.1–100/Mb. This variation may be due to key biological factors, such as UV exposure in melanomas, tobacco in lung cancer, viral origin in head and neck, or altered mismatch repair in colon cancer. In general terms, these factors leading to higher mutational densities correlate with a worse clinical outcome. In PTC, the few patients that have the highest mutation densities (>1/Mb) were at the highest risk of recurrences and this association remained after correction for age (14). These patients were enriched for mutations associated with the APOBEC process. The APOBEC family comprises cytidine deaminases that are innate immunity enzymes restricting propagation of retroviruses and retrotransposons. However, the presence of viral pathogens appears to be unlikely significant contributors to PTC pathogenesis according to TCGA. In addition, although radiation exposure is another plausible factor, PTC patients under such environmental condition were not reported to have higher mutation rates. Therefore, the causal factors contributing to an increased mutational rate in thyroid cancer remain to be elucidated.

In addition to total mutation rates, the mutational spectrum in each tumor is a source of heterogeneity that may help to reveal insights regarding causal factors. Gastrointestinal tumors (esophageal, colorectal, and gastric) show extremely high frequencies of transition mutations at CpG>T dinucleotides, which may reflect higher methylation levels in these tumor types. Although thyroid cancer showed no significant predominant type of mutational spectrum, it showed relatively high frequency of transition at CpG dinucleotides (18). However, its significance or functional implications remain unclear.

Figure 2
Non-synonymous mutation per tumor type. Rate of genetic mutations across the cancer genome ranges from low mutation in pediatric and liquid (leukemia) tumors to high mutation in solid tumors being the highest rate in mutagen-induced malignancies such as melanoma and lung. Papillary and anaplastic thyroid tumors (red lines) are in the extreme of solid tumors with low and high mutational rate respectively. The numbers in the bars are the number of non-synonymous mutations described in each tumor.
Genetic mutations and somatic copy number alterations (SCNAs)

Cross tumor analysis of multiple cancer types from TCGA has determined that a limited number of genetic alterations are responsible for most cancer subtypes (17). These alterations, no matter what tissue they come from, fall into two general categories of "oncogenic" signatures: genetic mutations and somatic copy number alterations (SCNAs). This distinction is based on the observation of an inverse relationship between the number of recurrent copy number alterations and the number of somatic mutations, which is clearest at the extremes of genomic instability. In other words, tumors have either a large number of somatic mutations or a large number of SCNAs, never both. This trend has been called the cancer genome hyperbola and tumors are positioned along the two axes of this hyperbola. About 30 subclasses of tumors have been characterized according to these oncogenic signatures in a tissue-independent manner. Under such premise, clinicians are beginning to imagine a future where cancers may be described by their mutations, such as an ERBB2-amplified tumor or a PI3K pathway-mutant carcinoma, in addition to the clinical and pathological data based on the tissue of origin.

As expected, PTC tumors were among those dominated by somatic mutations affecting mostly the RTK-RAS-RAF pathway. Integrating genomic data from 18 cancer types from TCGA with 3299 tumor samples including 400 samples of PTC, the study by Ciriello et al. showed that PTCs were within the class of tumors with primarily somatic mutations (see supplementary data in (17)). Moreover, thyroid tumors emerged separately as a small subgroup strongly characterized by BRAF mutations. The majority of the genetic alterations observed in almost all samples of almost all tumor types encode components of at least one of these four major oncogenic pathways: RTK-RAS-RAF, PI3K-AKT-mTOR, cell cycle, and p53-DNA repair. Somatic mutations in PTC fit in one or more of such 30 oncogenic signatures preferentially within the RTK-RAS-RAF oncogenic pathway and, along with other cancer types, make all of them sensitive to the same targetable actions. In addition, whereas PTC has a relatively high proportion of somatic mutations and a relatively low proportion of SCNAs (around 30%), a similar proportion seems to be maintained in PDTC and ATC (around 40%) (15, 16). Somatic mutations in PDTC/ATC seem to affect preferentially not only RTK-RAS-RAF (similar to PTC) but also the PI3K-AKT-mTOR and p53-DNA repair pathways. Altogether, this suggests that both well-differentiated carcinomas (PTC and most likely FTC) and PDTC/ATC are dominated by somatic mutations.

Making this distinction may have important clinical implications as these alterations are directly or indirectly therapeutically targetable, given the current availability of anticancer drugs.

Although all forms of thyroid cancer show a relatively small fraction of SCNAs compared with somatic mutations, determining how SCNAs promote thyroid cancer is an important challenge. One third of PTCs were found to have SCNAs and the majority of these PTCs have no concurrent driver mutations or fusions, suggesting that these alterations are oncogenic driver events (14). This trend persists in PDTC and ATC, where SCNAs were more frequent in those patients lacking driver mutations. SCNAs have critical roles in activating oncogenes and in inactivating tumor suppressors and the region affected by SCNAs often encompass many genes. One of such regions, the isolated loss of 22q, has attracted much attention. It affects 10% of PTCs, the majority of which encompass the follicular variant, which are enriched for RAS mutations. In fact, loss of 22q is present in 45% of RAS-mutant PTC, particularly HRAS (19). Similar to follicular variant of PTC, PDTC also has a relatively high frequency of loss of 22q (22%) and was also preferentially associated with RAS. By contrast, BRAF-mutant tumors or ATC had barely any loss of 22q. This region at 22q includes NF2 and CHEK2, known to be tumor suppressor genes. Inactivation of the tumor suppressor NF2 has been shown to increase mutant RAS signaling resulting in higher MAPK intensity and leading to PDTC in transgenic mice (19), illustrating how SCNAs and somatic mutations cooperate to induce tumor progression. A second region affected by SCNAs, gain of 1q, is present in around 15% of PTC and is associated with BRAF mutations and higher risk of recurrence, yet no candidate genes have been found so far (14). Gain of 1q was also associated with a worse survival in PDTC (16). Lastly, 8p and 17p losses along with 20q gains were far more frequent in ATC genomes, being 20q gains associated with worse survival (16). Overall, SCNAs are not predominant in thyroid cancer compared with somatic mutations, but they participate in essential ways in tumor initiation and progression of some patients. There is a need to identify the gene targets of driver SCNAs (which often encompass many genes) and elucidate their functional roles in order to widen the horizon of targetable actions.

New cancer genes and “the long tail”

Cancer genomics shows that for many cancer types, a handful of cancer genes are mutated at high frequency,
but many more cancer-related genes are found mutated at much lower frequencies. This collection of low-frequency mutations has been termed the “long tail” (12) (Fig. 3). In PTC, 80% of the driver events, mostly somatic point mutations and fusion events, were concentrated in four genes (BRAF, NRAS, KRAS, and RET) and the remaining were dispersed across at least 31 genes (14). Many tumor types exhibit similar “long-tail” distributions and some of the genes found mutated at low frequencies in some cancers are more commonly and significantly mutated in other cancers. Many of the new cancer genes related to thyroid cancer belong to this long tail, and despite its low frequency, a complete catalog of mutations in this long tail will be essential for recognizing dysregulated pathways and optimal targets for therapeutic intervention, paving the way to establish the so-called precision medicine.

One of these low-frequency genetic drivers that are statistically significantly mutated in the PTC genome is EIF1AX (14). Mutations in EIF1AX are present in 1% of PTCs and were mutually exclusive with mutation in MAPK genes, suggesting that this genetic alteration is a new tumor-initiating driver event. EIF1AX is a ribosomal protein involved in protein translation and seems a promising and intriguing cancer gene. In 2013, targeted resequencing of uveal melanoma (the most frequent malignant tumor of the eye) showed that 77% of the tumors with disomy 3 had mutations in either EIF1AX (48%) or SF3B1 (29%), a protein essential for pre-mRNA splicing, being mutations in both genes mutually exclusive (20). Moreover, individuals with SF3B1- and EIF1AX-mutant tumor had better prognosis and were more frequent among males with uveal melanoma. The mechanism through which EIF1AX exerts its oncogenic action is unclear. EIF1AX encodes eukaryotic translation initiation factor 1A (eIF1A), which stimulates the transfer of methionyl initiator tRNA (Met-tRNAi) to the small (40S) ribosomal subunit (21). EIF1AX mutations are thought to diminish the rate of bulk translation and might in turn induce transcription factors whose mRNA translation is inversely coupled to ternary complex. The near-mutual exclusivity of EIF1AX alterations with MAPK pathway mutations together with recurrent mutations in other tumors (20) suggests that EIF1AX is a novel cancer gene involved in PTC initiation. In addition, EIF1AX mutations have been recently shown to have a higher prevalence in PDTC and ATC (around 10% in both), particularly in RAS-mutant cases (15, 16). This intriguing coexistence between RAS and EIF1AX suggests that this ribosomal protein is also an important contributor to tumor progression and an attractive new target for further investigation (Fig. 1B).

In addition to EIF1AX, mutations in CHEK2 and PPMID, involved in DNA repair, were also low-frequency genes found to be significantly mutated genes and occurred concomitantly with MAPK pathway mutations, suggesting that these genetic alterations are new late genetic events (14). Moreover, although not statistically significant, there were eight additional DNA repair-related mutations in 6.5% of the tumors, all mutually exclusive. CHEK2 normally functions in preserving genome stability through facilitating the accurate repair of DNA double-strand breaks (22). Located on chromosome 22q12.1, CHEK2 kinase is activated by ataxia telangiectasia mutated
(ATM) and phosphorylates TP53 and BRCA1, which activates the homologous recombination repair pathway. Thus, CHEK2 kinase acts as a tumor suppressor promoting genomic stability. Inactivating mutations in this gene have been found to be present in many tumor types such as breast, prostate, and ovarian cancers. In addition to mutations in CHEK2, 10% of PTC shows somatic copy-number alterations characterized by isolated loss of 22q, a region that includes CHEK2. Thus, genetic alterations in CHEK2 may not be so infrequent. The second gene to be significantly mutated, PPM1D, is a phosphatase that suppresses p53-mediated transcription and apoptosis through negative regulation of p38 MAP kinase (23). This gene is located in a chromosomal region known to be amplified in breast cancer. Finally, tumors carrying mutations in DNA repair-related genes had a significantly higher median mutation density and were associated with high-risk patients. These findings, together with the fact that occurred concomitantly with MAPK pathway mutations, suggest that acquisition of a defect in DNA repair is a late genetic event and represents a mechanism for progression to aggressive forms of PTC (Fig. 1B).

Many genetic alterations at low frequency observed in the genomic analysis of PTC were not statistically significant (14). Determining which genes show significantly more mutations than random expectation is a key step to detect which of these low-frequency genes is really a “driver” event and not a “passenger” event. A “driver” event is one conferring a selective growth advantage, but in the field of cancer genomics, it is more difficult to distinguish between “driver” events that are causally related to the development of cancer and random “passenger” events that have simply accumulated over the course of development and cell growth. It has been estimated that a complete catalog of mutations may be achieved with 600–5000 samples per tumor type, depending on background mutation rate. Despite this limitation, non-statically significant/low-frequency genes are of major interest as they encode for proteins involved in pathways and functional groups known to play a role in carcinogenesis. In the PTC genome, 20% of tumors showed rare mutations within epigenetic regulatory genes, being mutations in MLL (1.7%), ARID1B (1.0%), and MLL3 (1%) the most frequent (Fig. 3). Additional candidate drivers such as APC, ATM, NF1, p53, and SPOP were also observed at very low frequency. Also, there were rare fusion events affecting genes known to be important in other cancers: ALK (0.8%) including EML4/ALK (present in lung adenocarcinoma), FGFR2, MET, THADA, and LTK. Altogether, these low-frequent genetic events are involved in important pathways and functional groups such as chromatin remodeling, PI3K, WNT, and tumor suppressor genes and characterizing this 20% of patients with low frequent “driver” events would be essential to complete the whole spectrum of patients that may benefit from target-specific agents. Also, some of these rare events in the initial steps are more frequent in the late steps occurring during progression to PDTC/ATC (15, 16) (i.e., EIF1AX, p53, and chromatin-remodeling SWI/SNF).

**TERT promoter mutations: the paradigm of oncogenic mutations in non-coding DNA**

Cancer genome studies have largely focused on the exome rather than on the whole genome for reasons of cost and inadequate analytical techniques (12). At present, one feasible approach to study the whole genome and its vast extension of non-coding sequences is to focus on regions corresponding to known biological functions such as regulatory regions or promoters. Recently, somatic mutations in non-coding sequences, specifically in regulatory regions, have been shown to be suggestive of a general mechanism for oncogene activation in several types of cancer. The recent identification in melanoma of activating mutations in the regulatory region of TERT is a paradigmatic example (24, 25). In an effort to analyze somatic mutations in the regulatory regions of 505 tumor genomes across 14 cancer types, Fredriksson and coworkers showed that TERT promoter mutations were found in six of the 14 cancer types studied: glioblastoma (62%), low-grade glioma (61%), melanoma (55%), bladder carcinoma (48%), thyroid carcinoma (18%), and lung adenocarcinoma (3%) (26). In addition to TERT, the analysis showed other oncogenic promoter mutations, yet with a much lower frequency (26). One example is PLEKHS1, a largely uncharacterized protein containing a pleckstrin homology domain, a motif that is present in a range of signaling proteins, including AKT family members. Interestingly, thyroid carcinomas (6%) along with other cancer types such as bladder (29%), breast (4%), and lung (2%) had somatic mutations in at least one of two distinct non-coding positions, 70 and 73 bp into intron 1.

Mutations in the TERT promoter were first reported in melanomas and provided a new mechanism by which cells could acquire increased telomerase activity (24, 25). An important observation is that TERT promoter mutations showed a strong and genome-wide significant association with increased mRNA expression. However,
there was a notable variation in the association between mutations and mRNA levels across cancer types: only three cancers (glioblastoma, low-grade glioma, and thyroid carcinoma) contributed in a considerable way to the positive association being particularly striking in thyroid carcinoma. TERT promoter mutations co-occurred significantly with BRAF mutations in both melanoma and thyroid carcinoma (27, 28, 29) consistent with the observation that BRAF may activate ETS factors that bind the mutant TERT promoter. TERT transcription by somatic mutations involves de novo generation of binding sites for ETS-family transcription factors. However, an additional mechanism has been proposed. TERT promoter mutations may also control the expression of the nearby gene CLPTM1L. This gene, originally identified due its ability to confer cisplatin resistance in ovarian cancer, is located only ~22 kb from TERT on chromosome 5 and experimental data have shown that CLPTM1L promotes tumor growth (30). Thus, somatic mutations in TERT regulatory DNA could have additional consequences beyond TERT activation.

TERT promoter mutations are present in 9% of PTC and occurred concomitantly with MAPK-pathways mutations (14, 29). The C228T substitution is the most common (7%), and the C228A and C250T substitutions are less common (0.3% and in 2.1% respectively). In addition to PTC, TERT promoter mutations are also present in FTC (14%), PDTC (40%), and ATC (45–73%) (16, 29, 31). Interestingly, TERT promoter mutations were subclonal in the small subset of PTC that harbored them, whereas they were clonal in PDTC and ATCs, suggesting a selection during tumor development toward cells highly immortalized (16). In line with this, TERT mutations appear to confer tumors with a markedly aggressive behavior. In PTC, TERT mutations are associated with aggressive clinicopathological features and high risk or recurrence, particularly when coexisting with BRAF, suggesting a synergistic interaction between BRAF and TERT (32). In ATC, survival of patients harboring TERT promoter mutations was markedly decreased, particularly in cancers with coexisting mutations of BRAF or RAS. Similarly, TERT-mutated PDTCs developed more distant metastases and had a trend toward greater mortality. In addition, TERT and EIF1AX mutations did not overlap in RAS-mutant ATCs, consistent with alternate pathways toward progression to ATC. Overall, TERT is an example of somatic mutations in regulatory regions of the genome that represent an important tumorigenic mechanism for progression to the most aggressive forms of thyroid cancer (Fig. 1B).

**From cancer genes to signaling and differentiation: toward a molecular classification**

Cancer genes act together in various signaling regulatory pathways and protein complexes (17). Clustering of mutations on known signaling pathways provides the basis for the molecular classification in cancer. For long, it was clear that PTC is a MAPK-driven cancer, but researchers at the TCGA have made strides to determine that there are two different genetic types of PTCs: BRAF-like and RAS-like (Table 1 and Fig. 1B) (14). According to the consequences and intensity of the signaling through MAPK, the work detailed important differences between the two genetic types, particularly in promoting tumor development and dedifferentiation. The researchers developed a scoring system – the BRAF-RAS score (BRS) based on a 71-gene signature – to reflect gene expression in the two PTC types, allowing them to characterize tumors and determine both the pathway a tumor uses to signal and its relative differentiation. In addition, to assess the consequences in differentiation, the researchers also developed a thyroid differentiation score (TDS) based on the expression of 16 genes involved in iodide metabolism and thyroid function. Ultimately, where a tumor lies on the scale can have important treatment implications because different tumor signaling properties can mean the cancer responds differently to particular therapies.

The work detailed important differences between the two genetic types, particularly the way BRAF and RAS signal to promote tumor development and growth. As BRS
is a reference continuous scale, BRAF- and RAS-mutant tumors are positioned each one at both ends of the scale. BRAFV600E tumors have high MEK-ERK signaling as they do not respond to the negative feedback from ERK to RAF as BRAF signals as monomer and not as a dimer (33). In normal cells, ERK induces a negative feedback on effectors upstream in the pathway, resulting in impairment of RAF dimerization. At the other end, RAS tumors have low MEK-ERK signaling (as RAF dimers are preserved) and they also have concurrent activation of PI3K. Finally, the other less common mutations can be positioned along the scale according to the signaling consequences derived from BRS (Table 1).

Tumors with high MEK-ERK signaling (BRAF-like) result in less differentiated tumors (according to TDS) and in higher output of the ERK transcriptional programs. According to TCGA, this transcriptional output is particularly represented by dual-specificity phosphatases (DUSP) 4, 5, and 6. Paradoxically, these DUSPs are MAPK-specific phosphatases that attenuate MAPK-dependent (34). On the other hand, tumors with low MEK-ERK signaling, (RAS-like) have a surprisingly higher phosphorylation p90RSK, an ERK substrate that is associated with mTOR activation. RAS-like tumors also show activation of an antiapoptotic program, characterized by S112-BAD phosphorylation (a target of P90RSK) and BCL2 overexpression. These intriguing results need further investigation and it would be crucial to understand why these two members of the same pathway, when mutated, activate so differently the ERK transcriptional programs.

Eventually, it appears that BRAF and RAS tumors, although activating MEK-ERK pathway, follow very different signaling routes that may critically determine the way the tumors progress, disseminate, and acquire late genetic events. This is well illustrated in PDTCs, where BRAF-mutant PDTCs are smaller and have higher frequency of lymph node metastases, whereas their RAS-mutant counterparts are larger and have a higher rate of distant metastasis (16). Genetically speaking, RAS-mutant tumors strongly coexist with EIF1AX mutations, which are present in only 1% of PTCs but in approximately 10% of PDTCs and ATCs; on the other hand, BRAF-mutant tumors do not coexist with EIF1AX but are enriched with alterations in members of the PI3K/AKT/mTOR pathway (Fig. 1B). Moreover, BRS correlates with the BRAF/RAS status in PDTC.

Beyond this molecular classification based on two genetic types, BRAF-driven tumors have a broader range of genetic complexity than previously thought, with distinct subtypes. In contrast to RAS-driven tumors, BRAF tumors are very heterogeneous in terms of gene expression, microRNA profiles, and epigenetic alterations. This diversity fits with the significant variation in clinical outcome seen in patients harboring BRAF tumors. Interestingly, the genomic analysis showed that BRAF tumors could be grouped into different clusters (two based on DNA methylation data to five based on microRNA (miRNA) data), which did not overlap with each other. The clusters were significantly different based on parameters such as proportions of driver mutations and gene fusions, mutational densities, histological and risk profiles, and differentiation (Table 2). Importantly, because miRNAs are stable biological markers that can be detected in serum, cytological aspirates, or directly in tumors, we can expect that these findings may lead to clinically relevant subclasses based on miRNA expression as we will see in the next section. Thus, genomic analysis may serve as a very useful source to provide critical information regarding which miRNAs can be used as clinical meaningful markers and guide future studies.

**The shaping and functional consequences of microRNA landscape in thyroid cancer: deciphering regulatory circuits**

The role of microRNAs (miRNA) in fine-tuning gene expression has become a major regulatory mechanism involved in developmental and pathological processes such as cancer. The extent to which individual miRNAs regulate common processes of tumor biology across diverse cancer types is beginning to be known and new examples of miRNAs that coordinate regulation of cancer pathways (i.e., DNA demethylation pathway members) are emerging (35). Genomic analysis of PTC tumors in TCGA clearly suggests that miRNA expression patterns

**Table 2** miR clusters in PTC and its clinical relevance.

| Cluster 1 | miR-182, miR-183, miR-204 (high) |
| Cluster 2 | miR-142, miR-143 |
| Cluster 3 | miR-148a, miR-142 |
| Cluster 4 | Let7a, Let7f, Let7e, Let7b |
| Cluster 5 | miR-146b-5p, miR-146b-3p, miR-375, miR-221, miR-222, miR-204 (low) |
| Cluster 6 | miR-21, miR-221, miR-222, miR-204 (low) |

| RAS | Follicular variant | Low risk |
| BRAF | Classical | Low risk |
| BRAF | Classical and FV | Low risk |
| BRAF | Classical | Low risk |
| BRAF | Tall cell and classical | High risk |
define clinically relevant subclasses and may contribute to loss of differentiation and tumor progression. Huang and coworkers were the first to analyze the miRNA expression datasets of PTC from TCGA Data Portal (36). The authors emphasized that immune responses are significantly enriched and under specific regulation in the direct miRNA-target network. Later on, researchers at the TCGA analyzed the significance of miRNAs in the context of a multidimensional genomic analysis (14). Such analysis showed that six miRNA clusters could be defined in PTC (Table 2). Cluster 1 was enriched with miR-181 and miR-182 and was strongly associated with RAS-mutated tumors and the follicular variant. Within BRAF tumors, there were defined five miR clusters. Two of them, clusters 5 and 6, were associated with less-differentiated tumors and higher risk of recurrence. Cluster 5 is enriched with higher levels of miR-146b (both 3p and 5p strands) and miR-375 and low levels of miR-204. The vast majority of this subclass contained classical PTC and harbored the BRAFV600E mutation, present loss of differentiation, and high risk of recurrence. Cluster 6 was characterized by high levels of miR-21 and low levels of miR-204. Histologically, this cluster comprises the majority of the tall cell variant and also classical PTC, had the highest frequency of BRAFV600E mutation yet some RET fusions are also present, and were less differentiated and had high risk of recurrence. The other three clusters were associated with highly differentiated PTCs and were less aggressive.

Cluster 4 was significantly abundant for members of the Let7 family and cluster 2 for miR-142. Both clusters were enriched for BRAFV600E, fusions in RET and NTRK were also present. Whether miRNAs would help to differentiate low risk from high-risk BRAF-like PTC remains to be clarified in the future, but it looks as a promising field to explore. It is worth to underscore that microRNAs may serve as highly useful markers that can be detected in the serum, aspirates, and paraffin-embedded tumors. Thus, defining clinically relevant subclasses based on miRNA expression attracts much attention from a clinical point of view.

Apart from this clinically relevant miRNA expression patterns, the role of specific miRNAs in thyroid carcinogenesis is currently being clinically and experimentally well established. MiR-146b is currently one of the most studied miRNAs in PTC. MiR-146b appears to be a prognostic factor for PTC, as it is associated with aggressive clinicopathological features and a poor clinical outcome (37). It has also been proven to be elevated in the serum of patients with PTC and some of its target genes have been validated in thyroid cancer (38, 39). MiR-146b specifically represses PAX8 and NIS, two genes essential for determining the differentiate phenotype of thyroid cancer (40). Antagonizing miR-146b in human thyroid cancer cells has been shown to reinstate NIS-mediated iodide uptake (41). Apart from NIS, miR-146b is predicted to repress other iodide-metabolizing proteins such as DEHAL and DIO2 (40). It happens that NIS, DEHAL, and
DIO2 are downstream targets of PAX8 (42). Thus, miR-146b simultaneously represses the transcription factor and its downstream targets in what is called a feedforward loop (Fig. 4). This circuit functions to suppress leaky transcription and hence target genes will only be expressed whether the levels of the miRNA decrease (43). In addition, PAX8 regulates miR-146b transcription forming a regulatory negative feedback loop where PAX8 limits its own activity by inducing a repressor, miR-146b. Such a loop can result in coexpression of both components, either in steady-state levels or in oscillation (44). Therefore, the balanced expression of both components depends on additional input signals. For instance, an upstream signal may upregulate the miRNA which, in turn, represses all its direct targets including the TF and as a result, all downstream targets of the TF are also repressed. As a consequence, network circuits where miRNAs and TF are involved represent efficient mechanisms that allow the spreading of regulatory effects (Fig. 4) (45). Tumor cells, where miR-146b is highly up-regulated, may well be making use of such efficient mechanisms to aberrantly exploit them favoring dedifferentiation and progression.

At the genome scale level, it is well established that these regulatory circuits involving miRNAs and transcription factors are prevalent mechanisms of gene expression (44, 46). The regulatory circuit miR-146b-3p-PAX8-NIS described above illustrates the importance of such regulatory miRNA-TFs pairs as examples of regulators of essential biological functions like the metabolism of iodide and ultimately dedifferentiated state in thyroid cells (Fig. 4). But, presumably, miR-146b might not be the only one. Other potential regulatory circuits might be operating in both normal and tumor cells. MiR-182, predominant in RAS-mutated tumors, is predicted to repress PAX8 and DEHAL, and miR-375, more prominent in BRAF tumors, is predicted to repress both NKX2.1 and DEHAL (40). Thus, antagonizing miRNAs in thyroid cancer may disrupt these regulatory circuits that are efficient contributors of a less-differentiated state in the tumor cells and may represent a new strategy to redifferentiate tumor cells and increase iodide uptake.

**New steps in thyroid cancer genetic predisposition**

Although many of the genetic factors that drive a cancer are acquired through somatic mutation, some are inherited at birth. Epidemiological studies have long noted an increased risk of cancer in relatives of affected individuals (47). The estimated ratio for common cancers such as breast, lung, colorectal, and prostate is within the 2–4 range (48, 49). Of note, among the few cancers with values exceeding 4, PTC stands out with the highest of all ratios (FRR=8.48–12.42) (50). In recent years, genomics has revealed some genes that influence predisposition to thyroid cancer, although the picture remains far from complete (Table 3).

One way to identify genes that confer predisposition to cancer is to study rare, highly penetrant Mendelian cancer syndromes. These syndromes arise when mutant alleles confer such a high increased risk (>ten-fold) that it is straightforward to trace their transmission in families by linkage analysis. More than 100 genes underlying such cancer syndromes have been identified (e.g., BRCA1, BRCA2 in breast cancer or APC, MUTYH and the mismatch repair genes in colon cancer) and have been deeply informative about cancer biology (51). However, although linkage analyses in PTC have resulted in numerous candidate loci that have been carefully studied by positional cloning strategies (52), no high-penetrance gene has been convincingly found. In addition, pedigrees displaying Mendelian-type inheritance of PTC are very rare, and large pedigrees with more than five affected individuals are exceptional. Very recently, Gara et al.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type</th>
<th>SNPs</th>
<th>Possible mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOXE1</td>
<td>Low-penetration gene</td>
<td>9q22 rs965513, 9q22 rs1867277</td>
<td>Differentiation and migration</td>
<td>(59)</td>
</tr>
<tr>
<td>TSHR</td>
<td>Low-penetration gene</td>
<td>9q22 rs965513</td>
<td>Differentiation</td>
<td>(60)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14q13.3 rs2284734</td>
<td></td>
<td>(63)</td>
</tr>
<tr>
<td>PAX8 &amp; SKTB1</td>
<td>Gene-gene interaction</td>
<td>SNP pair rs4848323 rs1378624</td>
<td>Differentiation and proliferation</td>
<td>(63)</td>
</tr>
<tr>
<td>miR-146a</td>
<td>Small non-coding gene</td>
<td>5q33.3 rs2910164</td>
<td>Proliferation</td>
<td>(67)</td>
</tr>
<tr>
<td>PTSCC1</td>
<td>Long non-coding gene</td>
<td>8q24</td>
<td>Tumor suppressor</td>
<td>(52)</td>
</tr>
<tr>
<td>PTSCC2</td>
<td>Long non-coding gene</td>
<td>9q22 rs965513</td>
<td>Tumor suppressor</td>
<td>(62)</td>
</tr>
<tr>
<td>PTSCC3</td>
<td>Long non-coding gene</td>
<td>14q13.3 rs944289</td>
<td>Tumor suppressor</td>
<td>(69)</td>
</tr>
<tr>
<td>HABP2 ?</td>
<td>Autosomal susceptibility gene</td>
<td>10q25.3 rs7080536</td>
<td>Tumor suppressor</td>
<td>(53)</td>
</tr>
</tbody>
</table>
reported that a germline missense mutation, G534E in HABP2, was responsible for the segregation of familial non-medullary thyroid cancer in a family and occurred in 4.7% of 423 patients with thyroid carcinoma in the TCGA database (53). However, segregation of this mutation has not been seen in larger series of familial non-medullary thyroid cancer (54, 55) and more evidence is needed to consistently support, or not, a role of HABP2 G534e variant in PTC. Therefore, it seems that thyroid cancer behaves as a complex disease where multiple genetic variants located on low-penetrance genes interact with each other and with environmental factors, thus modulating individual susceptibility (56, 57).

To identify low-penetrance genes that confer more modest risks, it is necessary to use population-based association studies rather than family-based linkage studies. The methodology for association studies depends on whether one wishes to study “common” (>1%) or “rare” (<1%) variants. Common variants are frequent enough that they can be tested for their individual effects on cancer risk by genotyping of millions of variants in cases and controls in “genome-wide association studies” (GWAS) (58). The first GWAS to screen a cohort of non-medullary thyroid cancer cases came from Iceland and the authors identified two SNPs located at 9q22 rs965513 and 14q13 that are strongly associated with increased risk of PTC and FTC (59). A second population-based association study in Spain (and validated in Italy) performed a candidate gene approach showing that a different variant within the FOXE1 gene (SNPs1867277) exhibits the strongest associated PTC susceptibility (60). Interestingly, in the study from Iceland, the closest gene to the top variant rs965513 at 9q22 is FOXE1. Although the association of rs1867277 to PTC shown in the Spanish study could be an independent association signal (61), it has been suggested that it could be explained by linkage disequilibrium with the variant rs965513 seen in the study from Iceland (62). The Spanish study further described the underlying mechanism involved. The variant within FOXE1 affected gene transcription through the differential recruitment of USF1/USF2 transcription factors. In addition, another variant affecting the TSHR gene is associated specifically with predisposition to the classic subtype of PTC (63). Other work has confirmed the FOXE1 polymorphisms and its association with familial and sporadic non-medullary thyroid cancer susceptibility (64), and interestingly, studies performed in different populations confirmed these data.

Although the number of identified low-penetrant genes for thyroid cancer has increased in recent years, many of the genetic components remain unexplained compared with other complex diseases. One way to identify new genes is through the assessment of gene–gene interaction. Although variants in single genes explain a relatively small proportion of cases, it is likely that common variants at different loci interact to modify susceptibility. When two or more genetic variants act together in a non-additive way, this gene–gene interaction is known as epistasis (65). One study explored the epistatic contribution of genetic factors to PTC susceptibility. Based on one of the largest series of thyroid cancer cases so far, the authors revealed a significant interaction between variants in PAX8 and STK17B (63), yet the mechanism through which these two gene variants interact remains unclear. Overall, it is worth to point out that FOXE1 and PAX8 are at the center of a regulatory network of transcription factors and cofactors that is essential for thyroid gland formation and the maintenance of the thyroid differentiated state in adults (66).

A second way to identify new genes is focusing on non-coding genes. A limited number of non-coding candidate genes have been described, such as a microRNAs (67) and large intergenic non-coding RNA (lincRNA) gene in chromosome 8q24 (52). The first SNP in conferring cancer susceptibility was shown for miR-146a in thyroid cancer (67). In an association study of 608 PTC patients and 901 controls, a marked difference in genotype distribution of the miR-146a SNP was found, the heterozygous state being associated with the risk of PTC. Thyroid cancer was thus the first among all cancers in which researchers demonstrated a role for a miRNA polymorphism in the predisposition to cancer. Subsequent results implicating inherited SNP were reported in breast cancer, hepatocellular carcinoma, and chronic lymphocytic leukemia (reviewed in (68)). Nevertheless, for thyroid carcinomas, the most abundant sequence variants have yet to be determined for all miRNA. Moreover, microRNA genetic variations can create new functional mature miRNAs by changing the structure of precursors and/or modifying the sequence of the “seed regions” responsible for targeting mRNA. The functional implications of these genetic variations and there target sites need to be explored.

Two long non-coding RNA genes (PTCSC1 in 8q24 and PTCSC3 in 14q13) have been reported as PTC susceptibility candidate genes (52, 69). Together with PTCSC2 in 9q22, it seems that a class of prototype non-coding regulatory RNA genes plays an important role in thyroid cancer predisposition. Decreased expression in PTC tumors is a common feature for the three non-coding RNAs (PTCSC1, PTCSC2, and PTCSC3), implying that these transcripts act as tumor suppressors. Moreover, the genotype of rs965513 was associated with expression levels of PTCSC2, FOXE1,
and TSHR in unaffected thyroid tissues, suggesting perhaps a multilayered regulatory network in the thyroid. It is logical to assume that decreased expression of FOXE1 and TSHR predisposes thyroid cells to dedifferentiate, an essential step toward malignant transformation. This is consistent with a repeatedly proposed theory that FOXE1 is involved in the pathogenesis of PTC (60, 70, 71). More work is needed to explore whether long non-coding RNA genes and thyroid differentiation genes interact with each other and by what mechanism they affect the transcriptional regulation of FOXE1 and TSHR.

Concluding remarks

Knowing the cancer genome is changing the way we look at some essential aspects of the molecular pathogenesis of thyroid cancer. For instance, such knowledge permits the reshaping of the multistep model of thyroid tumors and offers new opportunities for future research. It also points to new potential molecular markers with clinical utility in diagnosis and prognosis and, most importantly, new targetable actions based on new cancer genes, regulatory circuits, or biological processes associated with initiation and progression of thyroid cancer. Positioning thyroid cancer in the context of the human cancer genome will surely help us to better understand this disease in order to move forward toward a more precise and personalized clinical management and treatment strategies for our patients.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work did not receive any specific grant from any funding agency in the public, commercial, or not-for-profit sector.

Acknowledgements

We are grateful to the following institutions: Dirección General de Proyectos de Investigación (SAF2013-44709-R), Instituto de Salud Carlos III (RD12/0036/0030 and PI14/01980), Comunidad de Madrid (S2011/BMD-23), and Fundación Asociación Española del Cáncer (GCB14142311CRES) for supporting our work.

References


ETS-regulated negative feedback mediator of oncogenic ERK signaling and elevated transcriptional output of the pathway. (V600E)BRAF is associated with disabled feedback inhibition of RAF-recurrence. (doi:10.1210/jc.2013-2383)

Ghossein RA & Fagin JA. Frequent somatic TERT promoter mutations promote oncogenic RAS-induced thyroid cancers via YAP-dependent protein synthesis. (doi:10.1210/jc.2013-2383)

El-Naggar AK & Xing M. Highly prevalent TERT promoter mutations in familial and sporadic melanoma. (doi:10.1126/science.1229259)

Moll I, Nagore E, Hemminki K. Modification of cancer risks in offspring of relatives of cancer probands. (doi:10.1083/jcb.201210031)

Voza F, Smith VE, Ganly I, Taylor BS, Persaud Y. Prognostic implications of miR-146b expression and its functional role in papillary thyroid carcinoma. (doi:10.1093/jnci/djt235)