REVIEW

Gene expression profile of human thyroid cancer in relation to its mutational status

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Abstract

This review describes the gene expression profile changes associated with the presence of different mutations that contribute to thyroid cell carcinogenesis. The results are discussed in the context of thyroid cancer biology and of the implications for disease prognosis, while the diagnostic aspect has been omitted. For papillary thyroid cancer (PTC), the most characteristic gene expression profile is associated with the presence of *BRAF* mutation. *BRAF*-associated PTC differ profoundly from RET/PTC or RAS-associated cancers. Simultaneously, they retain many characteristic gene expression features common for all PTCs, induced by the alternative mutations activating MAPK pathway. Although the difference between papillary and follicular thyroid cancer (FTC) is significant at the gene expression profile level, surprisingly, the RAS-related signature of FTC is not well specified. *PAX8*/peroxisome proliferator-activated receptor γ (*PPAR* γ) rearrangements, which occur in FTC as an alternative to the *RAS* mutation, are associated with specific changes in gene expression. Furthermore, the difference between well-differentiated thyroid cancers and poorly differentiated and anaplastic thyroid cancers is mainly a reflection of tumor degree of differentiation and may not be attributed to the presence of characteristic mutations.

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Introduction

Gene expression profiling of thyroid tumors has expanded our knowledge of the molecular biology of thyroid cancer. Several recent reviews addressed the biological and diagnostic aspects of microarray-based studies of thyroid cancer (Eszlinger *et al.* 2007, Handkiewicz-Junak *et al.* 2010, Kouniavsky & Zeiger 2010). However, this field remains open and new reports continue to appear, thus, updating its present status is necessary. The aim of this review is to describe the gene expression profile changes associated with different mutations that contribute to thyroid carcinogenesis (see Kondo *et al.* (2006) for an overview of genetic alterations involved in the development of thyroid cancer). The results will be discussed in the context of thyroid cancer biology and of the implications for disease prognosis, while the diagnostic aspect will be omitted. Only follicular cell-derived neoplasms will be considered and emphasis will be placed on papillary thyroid cancer (PTC), the most frequent histotype, since it has been the subject of the majority of microarray-based studies published to date.

Transcriptome of papillary thyroid cancer

Papillary thyroid cancer develops directly from the thyroid cells and does not have a benign intermediate. The majority of PTCs exhibit the *BRAF* V600E activating point mutation, which is observed in 40–70% of cases, and is more frequently found in older patients with more aggressive disease (see Handkiewicz-Junak *et al.* (2010) for review). Chromosomal rearrangements involving receptor tyrosine kinase genes such as *RET/PTC* or *TRK* rearrangements, or rarely, activating *RAS* point mutations, are other molecular events involved in the activation of the MAPK–ERK cascade (mitogen activated protein kinases cascade) and in the development of PTC (Kimura *et al.* 2003, Fagin 2005).

The *BRAF* V600E mutation and the gene expression profile of papillary thyroid cancer

BRAF mutation is associated with the morphological and functional alterations characteristic of the PTC phenotype. The detection of *BRAF* mutation is a specific but not sensitive feature of papillary thyroid

cancer, since this mutation has not been observed in benign thyroid lesions (Eszlinger & Paschke 2010, Soares & Sobrinho-Simoes 2011). The most distinct phenotypic feature of BRAF-positive tumors is the presence of papillary structures that determine the diagnosis of the classical variant of PTCs (Nakamura *et al.* 2005, Xing *et al.* 2005, Frasca *et al.* 2008). Notwithstanding, this may be a secondary phenomenon. Jakubowski & Hunt (2009) compared the frequency of BRAF mutation in PTC showing a mixed papillary and follicular growth pattern and observed no difference, the BRAF mutation status was always concordant, in both areas showing papillary and follicular growth type, at the overall rather high BRAF mutation frequency.

Functionally, the presence of the BRAF oncoprotein results in the inhibition of membrane sodium–iodide symporter (NIS) expression. This is the most often evoked explanation for the clinically well-known tendency of BRAF-positive PTCs to be non-radioiodine avid, to have a more aggressive clinical course, and to have a poorer prognosis (Xing *et al.* 2005, Handkiewicz-Junak *et al.* 2010). However, a deeper insight into the molecular profile of PTC and the presence of different mutations reveals a more complex network of molecular interactions that should be further elucidated both on the basis of gene expression research and on the basis of prospective clinical studies. This controversy cannot be clarified without integrating PTC variants with tumor invasiveness (Soares & Sobrinho-Simoes 2011). In addition, without neglecting the role of the BRAF mutation in PTCs with poor prognosis, there are many other genetic alterations known to be associated with the progression of papillary thyroid cancer to more aggressive phenotypes, including mutations of TP53 (Fagin *et al.* 1993, La Perle *et al.* 2000), under-expression of E-cadherin, or dysregulation of catenin signaling (Karim *et al.* 2004).

The first study devoted to the comparison between PTC with BRAF mutations or RET and TRK rearrangements was published by Frattini *et al.* (2004), who stressed similarities in the gene expression profiles of PTC with various mutations. These authors were the first to detect a difference in the gene expression between BRAF-positive PTCs and those with receptor tyrosine kinase-related rearrangements (Frattini *et al.* 2004). Giordano *et al.* (2005) showed that the gene expression profile of the BRAF-associated PTC differs profoundly from the RET/PTC or RAS-associated PTC. Using a significance criterion of $P < 0.01$, these authors found over 3800 genes differentially expressed in PTC with BRAF compared to those with RET alterations. They also indicated that tumor mutational status was more strongly correlated with gene expression profile than its morphology. In opposition to Frattini *et al.* and Giordano *et al.* hypothesized that these mutations signaled through alternative pathways and might be

related to tumors with discrete mutation-specific phenotypic and biological features. One of the groups of genes most distinctly over-expressed in BRAF-mutated PTCs was related to the regulation of the immune response (TM7SF4, CLECSF2, STAT1, and LY75). The proposed unique role of the BRAF mutation in initiating a specific immune response in PTC was supported by further SAGE data (Oler *et al.* 2008). Comparing the BRAF status in a set of metastatic and non-metastatic PTCs with their gene expression profiles, Oler *et al.* found that cystatin 6 (CST6) and the chemokine gene CXCL14 were over-expressed and positively correlated with the BRAF V600E mutation, which was in agreement with the data of Giordano *et al.* Both CST6 and CXCL14 are putative downstream genes in the BRAF/MEK/ERK signaling pathway, CXCL14 being involved in the homeostasis of macrophages. Furthermore, the *in vitro* studies of Mesa *et al.* (2006), who focused on the early molecular consequences of different mutations leading to PTC, showed that BRAF-induced genes included many involved in the immune response, mostly in the innate response, such as the chemokine (C–C motif) ligands 2, 7, 15, and granulocyte–macrophage colony-stimulating factor 2 (MCP1, MCP3, CCL15, and GM-CSF respectively). In this study, a preferential induction of MMP3 and MMP9 and the exclusive activation of MMP13 were observed in PCCL3 cells with the BRAF mutation compared to a cell line with RET/PTC rearrangement. The authors related these differences to ERK activation, which was much higher because of the BRAF mutation. The association of MMPs (matrix metalloproteinases), which are able to degrade all components of the extracellular matrix, with the invasive phenotype and poor prognosis is well known in PTC (Klopfleisch *et al.* 2011, Yang *et al.* 2010, Horndler *et al.* 2011). The activation of BRAF in PCCL3 cells downregulated the mitochondrial respiratory chain (MRC) complex 1 genes. There are contradictory data in the literature concerning the influence of the inhibited MRC complex 1 genes on cell survival, but the most predominant studies indicate that it protects the cells from apoptosis (Mesa *et al.* 2006).

Although Giordano *et al.* (2005) identified many BRAF-related genes showing differential expression between RET/PTC-positive or RAS-positive PTCs, these authors did not analyze other factors that could differ in the population of BRAF-positive and BRAF-negative tumors that could cause gene profile differences (e.g. age, disease stage, or other still unknown mutations that BRAF may co-segregate with). In addition, an independent validation of the BRAF-related gene list was not performed. The multifactorial nature of gene expression changes in malignant tumors, which is well known from the studies of other cancers (Rhodes *et al.* 2004), underscores the need for multivariate analysis of the association between gene expression profile and different mutations. In univariate

approaches, the risk of incorrectly attributing an observed change in gene expression to the presence of the *BRAF* mutation is high. Our own analysis of the *BRAF*-related gene expression profile, which was performed on 49 PTC patients, confirmed the differences between *BRAF*-positive and both *RET*/*PTC*-positive and all no-*BRAF* cancers; the meta-analysis of our and Giordano's study is presented in Fig. 1. Surprisingly, a comparison of the genes selected on the basis of our study and those listed by Giordano *et al.* showed only 11 common genes in the *BRAF* versus *RET* comparison (D Rusinek, M Oczko-Wojciechowska, M Kowalska, M Świernak, M Jarzab, A Czarniecka, J Wloch, S Szpak-Ulczo, E Chmielik, D Lange, M Kowal, M Wiench, D Handkiewicz-Junak & B Jarzab 2007, unpublished observations). This lack of overlap clearly indicates that large numbers of cases are needed for multivariate analysis.

Currently, the only multivariate analysis carried out considering the influence of the *BRAF* V600E mutation on *PTC* gene expression was performed by Jo *et al.* (2006). Upregulation of the vascular endothelial growth factor (*VEGF*) gene was observed in *BRAF*-positive PTCs even after adjustment for extrathyroidal PTC invasion, tumor stage, and nodal and distant metastasis ($P=0.03$). The authors concluded that the over-expression of *VEGF* in association with the *BRAF* mutation might account for the high rate of recurrences and aggressive phenotype of PTC as well as its poor outcome.

Platelet-derived growth factor (*PDGF*; Wang *et al.* 2008) is among the highly upregulated genes in papillary thyroid carcinomas induced by the *BRAF* V600E mutation, and its over-expression in thyroid neoplasms (Yano *et al.* 2004) and its presence in other tumors (Terrile *et al.* 2010) have been reported earlier. The receptor for *PDGF* – *PDGFRB* – is able to activate the *ERK1/2* signaling through cross talk with other *PDGFRB*-induced pathways (Jurek *et al.* 2011). Moreover, Wang *et al.* (2008) observed a correlation between the presence of the *BRAF* V600E mutation and high platelet count, which is similar to the *BRAF* mutation itself, and was significantly associated with extrathyroidal invasion.

Many other genes were also associated with the presence of mutated *BRAF* Franzoni *et al.* (2009) showed that prohibitin (*PHB*) was over-expressed only in PTCs harboring the *BRAF* mutation. Transfection of a *BRAF*V600E construct resulted in an increase in *PHB* promoter activity and higher expression of the protein product. *PHB* may act as tumor suppressor or exhibit a permissive influence on tumor growth.

In their search for *BRAF*-related transcriptome changes, Nucera *et al.* (2010) attempted to identify a PTC progression-related gene signature. Using a gene set enrichment analysis, they selected 18 gene sets significantly associated with the presence of

Figure 1 Comparison of gene expression profiles of *BRAF*(+) PTCs to *RET*(+) PTCs (analysis performed by combining our data and those of Giordano *et al.*).

BRAF mutation and identified one set of down-regulated genes involved in cell polarity, indicating the importance of *BRAF*V600E in cell dedifferentiation. Comparison of *BRAF*-positive PTCs with controls led to the identification of many extracellular matrix genes related to the presence of *BRAF*. In their study, Nucera *et al.* focused on thrombospondin 1 (*TSP1*), a multi-functional matricellular protein that binds to many integrins and other matrix proteins, one among them being fibronectin 1, which is abundant in tumor stroma. Knockdown of the *BRAF*V600E gene led to a decrease in the *TSP1* expression that was associated with a reduction in the migration/invasion phenotype of tumor cells both *in vitro* and *in vivo*.

The mechanism of cancer invasion in PTC is not well understood. Riesco-Eizaguirre *et al.* (2009) concluded that PTC invasion, which is more distinct in *BRAF*-positive PTCs, is TGF β /Smad dependent. Gene expression analysis (Vasko *et al.* 2007) of microdissected intratumoral samples from central and invasive regions of invasive PTCs and normal thyroid tissue showed an upregulation of TGF β , *NFKB*, integrin pathway members, and *CDC42* in the invasive regions. Interestingly, the downregulation of a subset of mRNAs encoding proteins related to the epithelial to mesenchymal transformation (EMT) and involved in cell-cell adhesion and communication was observed in invasive PTCs. Vasko *et al.* proposed an association between *CDC42*/PAK signaling and the mechanisms of PTC invasion. In opposition to their results, Watanabe *et al.* (2009) performed a QPCR analysis to show a positive correlation between the *BRAF* V600E mutation and over-expression of vimentin, which was confirmed using NPA cells (inhibition of *BRAF* expression decreased vimentin levels). These authors also showed the *BRAF* V600E-dependent upregulation of fibronectin and *CITED1*, both of which have been identified as PTC markers. The role of fibronectin 1 as a biomarker was first shown by QPCR and immunohistochemistry (Takano *et al.* 1998) and later by microarray analysis (Huang *et al.* 2001, Jarzab *et al.* 2005), while the role of *CITED1* as a biomarker was first shown by microarray analysis (Huang *et al.* 2001) followed by immunohistochemistry (Prasad *et al.* 2004). Fibronectin 1, an adhesion-related gene, is considered a part of the EMT signature (Huang *et al.* 2001). CBP/p300-interacting transactivators with glutamic acid (E)/aspartic acid (D)-rich C-terminal domain (*CITED1*) is a transcriptional coactivator initially identified in melanoma and known to interact with *SMAD4C*, *CPB/p300*, and the estrogen receptor (de Caestecker *et al.* 2000, Yahata *et al.* 2000).

In this context, it is noteworthy that mutations of the *BRAF* gene, the V600E mutation being the most frequent, are not the only characteristics of PTC. They are found in about 60% of melanomas (also in benign nevi), in a subset of lung, ovarian, and colon cancers

and in childhood astrocytomas (Thomas 2006, Estep *et al.* 2007, Pratilas *et al.* 2008, Yousem *et al.* 2008, Farina-Sarasqueta *et al.* 2010, Schiffman *et al.* 2010). Interestingly, in nearly all these tumor localizations, the presence of the *BRAF* mutation is related to a rather better prognosis. Comparative studies of the *BRAF* gene expression signature in various tumor types may help to elucidate the relationship between the transcriptional consequences of its presence and the invasive tumor potential.

The miRNA expression profile of papillary thyroid cancer and its relation to *BRAF* mutations

Differences in gene expression profile related to the mutational status of PTC may be visible not only at the mRNA level but also at the level of the non-coding RNAs. In this context, Yoon *et al.* (2007) identified a novel non-coding RNA gene called *NAMA*, which is associated with the MAP kinase pathway and growth arrest, physiologically expressed in the testis and under-expressed in the *BRAF*-positive PTC.

Several authors have examined the differences in the levels of miRNAs between *BRAF*-positive and *BRAF*-negative PTCs and have shown contradicting results. Cahill *et al.* (2007) reported a unique miRNA expression signature in the cell lines with *BRAF* mutation in comparison to the normal thyroid cell line, showing 15 upregulated miRNAs and 23 down-regulated miRNAs. Sheu *et al.* (2009), however, did not detect any significant differences between *BRAF*-positive and *BRAF*-negative human PTCs, although their study was limited to a subset of five miRNAs. Chou *et al.* (2010) compared the levels of three miRNAs known to be over-expressed in PTC. A significantly higher expression of miR-146b was detected in *BRAF*-positive PTC, while miR-221 and miR-222, also shown to be related to a more aggressive tumor behavior, did not exhibit any difference in comparison to *BRAF*-negative tumors. Aherne *et al.* (2008) analyzed different fragments of one multifocal thyroid cancer and described miRNA profiles distinguishing tumors containing the *BRAF* mutation from other tumor types (with insular or anaplastic phenotype).

RET/PTC rearrangements and their relation to gene expression in PTC

RET/PTC rearrangements arise from the fusion of the 3' end of the *RET* gene that encodes the tyrosine kinase domain with the 5' domain of one of the several constitutively expressed genes. To date, 12 fusion genes and 15 different *RET*/PTC rearrangements have been described (Mishra *et al.* 2009), and the *RET*/PTC1 and *RET*/PTC3 are the most frequent rearrangements.

RET/PTC1 and *RET/PTC3* are paracentric inversions because both *RET* and its respective fusion partner *H4* or *NCOA4* (also called *ELE1*; *RFG* and *ARA70*) are localized on the long arm of chromosome 10 (Grieco *et al.* 1990, Santoro *et al.* 1994). *RET/PTC3* rearrangements were initially thought to be related to radiation-induced thyroid cancer (Rabes *et al.* 2000, Maenhaut *et al.* 2011). Now it is clear that they are characteristic of short latency PTC that develops in young persons, while *RET/PTC1* prevails in long latency PTC (Williams *et al.* 2004). In fact, *RET/PTC* rearrangements are quite frequent both in radiation-induced and in sporadic pediatric PTC. They are found in 50–70% of cases of children with sporadic PTC, while they are rare in older PTC patients, especially after the 45th year of age. The unique *RET/PTC*-related gene expression profile is less well characterized than that related to the *BRAF* status. Frattini *et al.* (2004) were the first to identify a subset of genes differentially expressed in PTCs carrying receptor tyrosine kinase-related rearrangements by analyzing *RET/PTC*- and *NTRK1*-mutated PTCs and comparing them to tumors with *BRAF* mutation. Giordano *et al.* (2005), who constructed a simple multigene classifier, were able to recognize an additional *RET/PTC*-positive tumor sample without knowing its mutational status by its gene expression profile only. The gene expression signatures that differed in *BRAF*, *RET/PTC*, and *RAS* tumors included genes whose changed expression was a direct consequence of mutation (of *RET/PTC* rearrangement) as well as other genes involved in the immune response, signal transduction, and other processes. The hypothesis that different downstream pathways are activated by *RET/PTC* was supported by Miyagi *et al.* (2004), who used cell culture experiments to show that *RET/PTC3* mutants signaled preferentially through the PI3K pathway compared to MAPK. Among the genes involved in signal transduction, besides *ERBB3*, *MET*, or *DAPPI*, *VAV3* deserved attention as a phosphoinositol-3-kinase pathway-related gene responsible for *AKT* activation that was preferentially expressed in PTCs with *RET* and *RAS* alterations.

Interestingly, the above-mentioned study of Mesa *et al.* (2006) on the early consequences of the *BRAF* V600E mutation and *RET/PTC3* rearrangement activation in rat thyroid PCCL3 cells revealed that the number of *RET/PTC3*-dependent genes was almost twice as high as that of genes activated by the *BRAF* mutation. In total, only 25% of genes, all related to the MAPK pathway, were co-regulated by these two genetic alterations. Among those genes regulated by *RET/PTC* that did not need the activation of the *BRAF* gene, there were many genes associated with the immune response, including the IFN (interferon) pathway genes. This was not the conclusion in Giordano's paper or in our own *BRAF*-related gene expression analysis of PTC (D Rusinek, M Oczko-Wojciechowska, M Kowalska,

M Świernak, M Jarząb, A Czarniecka, J Włoch, S Szpak-Ulczo, E Chmielik, D Lange, M Kowal, M Wiench, D Handkiewicz-Junak & B Jarząb 2007, unpublished observations), but the number of *RET/PTC*-dependent genes could be underestimated because of the lower number of *RET/PTC*-positive than *BRAF*-positive tumors.

Expression of thyroid-specific genes in relation to different mutations in PTC

The expression of key genes involved in thyroid hormone biosynthesis is known to be deregulated in thyroid cancer compared to normal thyroid tissue. The downregulation of the TSH receptor (*TSHR*), *NIS*, apical iodide transporter (*SLC5A8*), thyroperoxidase (*TPO*), and thyroglobulin as well as impaired *NIS* trafficking to the cell membrane was observed in papillary thyroid carcinomas, both with and without the *BRAF* mutation (Durante *et al.* 2007, Espadinha *et al.* 2009b). Simultaneously, the degree of dedifferentiation was higher in the *BRAF*-positive tumors. The loss of thyroid hormone production ability in PTC with the *BRAF* mutation was accompanied by the increased expression of the glucose transporter 1 (*GLUT1*), which reflects the V600E potential to induce the dedifferentiation processes (Durante *et al.* 2007, Puxeddu & Moretti 2007).

When comparing the wild-type *BRAF* PTCs with papillary thyroid carcinomas harboring the V600E mutation, the *NIS* transcript levels in the *BRAF*-positive PTC were about five times lower than those observed in the *BRAF*-negative tumors, with even more distinct differences at the level of protein expression (almost ten times lower; Durante *et al.* 2007). Romei *et al.* (2008) observed that the number of *NIS*-positive cells was significantly lower in *BRAF*-positive tumors (53.5 vs 72.6%), while at the mRNA level, the difference was seen both in the *NIS* and in the *TPO* genes. Investigating the differences related to the *BRAF* mutation in PTC, Riesco-Eizaguirre *et al.* (2009) analyzed the *BRAF*-induced secretion of functional TGFβ, which repressed the *NIS* expression by the activation of Smad. This mechanism has been shown to be MAPK–ERK kinase independent. In the interpretation of these results, it is important that not only the expression level but also the localization of the transporter is crucial for its function. In both *BRAF*-positive and *BRAF*-negative PTCs, the expression of the *NIS* protein was mainly limited to the cytoplasm and was less observed at the cell membrane.

The lower *SLC5A8* expression in the *BRAF*-associated PTCs is due to gene methylation (Porra *et al.* 2005). This thyroid-specific gene, unlike pendrin and *NIS*, is not TSH dependent. It is involved in the passive transport of iodide from the thyrocytes to the follicle lumen and was shown to be downregulated (40-fold) in the classical variant of papillary thyroid carcinoma

(CV-PTC). The decreased expression of *SLC5A8* is a consequence of exon 1 methylation, which is seen in 90% of CV-PTC. This epigenetic change silencing *SLC5A8* is also observed in colon cancer, where it occurs in the early stage of cancer development (Li *et al.* 2003). In thyroid carcinoma, methylation of *SLC5A8* is related to the later stages of cancer progression. A significant correlation between the presence of the *BRAF* V600E mutation and silencing of the *SLC5A8* suggests that methylation occurs as a secondary event to *BRAF*-mutated initiation of the MAPK pathway.

Gene expression changes common to all papillary thyroid cancer cases

The differences in gene expression between primary PTC and normal thyroid tissue are significant and encompass thousands of genes (Jarzab *et al.* 2005, Fujarewicz *et al.* 2007). The rather homogenous gene expression profile of PTC surprised the authors of the first microarray-based investigation (Huang *et al.* 2001). However, it seems less unexpected if one considers that different mutations activate the MAPK–ERK cascade and cause similar downstream molecular and functional effects. Many gene expression changes triggered by neoplastic transformation are not only common to all PTC but are also seen in follicular thyroid cancer (FTC; Polanski *et al.* 2007) and adenocarcinomas in general or even in all malignant tumors (Rhodes *et al.* 2004, Polanski *et al.* 2007).

Transcriptome of FTC

The biology of FTC and its gene expression profile is much less known in comparison with PTC (Cerutti *et al.* 2003, Weber *et al.* 2005, Cerutti 2007, Hinsch *et al.* 2009). The underlying molecular alterations of FTC encompass either activating *RAS* mutations (present in most cases already on the adenoma level) or *PAX8*/peroxisome proliferator-activated receptor γ (*PPAR* γ) rearrangements, which are more frequent at the carcinoma level (Knauf *et al.* 2006, Placzkowski *et al.* 2008). The oncocytic variant of follicular carcinoma expresses a very characteristic gene expression profile related to the presence of abundant mitochondria (Barden *et al.* 2003, Finley *et al.* 2004b, Baris *et al.* 2005).

RAS-related changes in the gene expression of FTC and other thyroid tumors

RAS point mutations that activate the protein product are typical of follicular thyroid adenomas (FA) and FTC, showing a frequency range nearly 50% in both types of tumors. Because they are found at equal frequency in FAs

and FTCs, *RAS* mutations are considered an early event in follicular neoplasia (Garcia-Rostan *et al.* 2003). There is no unequivocal proof of a larger tendency of *RAS*-positive follicular adenomas to progress to follicular carcinomas, especially since nothing is known about the molecular events responsible for the further malignant transformation of a *RAS*-positive follicular adenoma. *PAX8*/*PPAR* γ rearrangements occur preferentially in *RAS*-negative follicular tumors (Nikiforova *et al.* 2003b).

Surprisingly, the *RAS*-related signature of FTC is not well specified, possibly because it is not easily detected at the mRNA level. The impact of the *RAS* mutation on the gene expression of thyroid cells was studied using the FRTL-5 rat thyroid epithelial-derived cell line infected with the Kirsten murine sarcoma virus carrying the *v-Ki-Ras* oncogene (Visconti *et al.* 2007). Genes that were found to be significantly altered in *RAS*-transfected cells were validated by QPCR and studied in thyroid cancer cell lines of follicular cell carcinoma origin (WRO), papillary carcinoma origin (FB-2, NPA, TPC-1, and B-CPAP), and from anaplastic cancer (FRO and ARO). Annexin A2 and *RALA* (v-ral simian leukemia viral oncogene homolog A, *RAS* related) were upregulated in the *RAS*-transfected rat cells and positively validated in all seven cancer lines. Dual-specificity phosphatase 1 (*DUSP1*) and small cell lung carcinoma cluster 4 antigen (*CD24*) were downregulated by *RAS* in rat cells and also in all studied cancer cell lines (Rodrigues *et al.* 2007a).

RAS mutations are found in 0–15% of PTCs (Vasko *et al.* 2003, Abrosimov *et al.* 2007, Hou *et al.* 2007), mostly as activating mutations of codons 12 and 13 of the *NRAS* gene, and showing higher incidence in follicular variant of PTC (Vasko *et al.* 2003, Zhu *et al.* 2003, Castro *et al.* 2006, Di Cristofaro *et al.* 2006, Goutas *et al.* 2008). *RAS* mutations have also been reported to be frequent in poorly differentiated thyroid cancer (PDTC; Garcia-Rostan *et al.* 2003, Riesco-Eizaguirre & Santisteban 2007, Volante *et al.* 2009). However, the distinct gene expression profile differences between PTC and PDTC are related to the grade of differentiation itself rather than to the presence of different initiating mutations (Fluge *et al.* 2006). However, Pita *et al.* (2009) reported similarities between the follicular variant of PTC and the *RAS*-mutated PDTC.

Gene signature of the *PAX8*/*PPAR* γ rearrangement

PAX8/*PPAR* γ translocations (*PAX8*/*PPAR* γ fusion protein, PFP) are present in 26–56% of FTCs and also in a number of FAs. This kind of translocation generates a fusion oncoprotein that acts as a dominant negative inhibitor of wild-type *PPAR* γ and has unique transcriptional activity when compared with wild-type *PAX8* or *PPAR* γ . Lacroix *et al.* (2005) revealed a pattern

of 93 genes that discriminated FTCs with and without translocation, which at the morphological level were indistinguishable. PPAR γ -dependent genes were over-expressed in FTCs bearing the translocation, including angiopoietin-like 4, aquaporin 7, or a potential target – FGD3. No differences in the expression of thyroid-specific genes were noted. In addition, Giordano *et al.* (2006) reported that the presence of this balanced translocation is associated with a specific gene expression signature, which is composed of known PPAR target genes involved in fatty acid metabolism such as carnitine/acylcarnitine translocase (*SLC25A20*) and acetyl-CoA acyltransferase 1 (*ACAA1*), amino acid and carbohydrate metabolism, and specific micro-RNA target genes. Among the top, 20% of genes highly upregulated in PFP-positive FTCs were genes detected on chromosome 3p (95 from 341 genes). Four miRNAs were strongly over-expressed in FTCs harboring *PAX8/PPAR γ* rearrangement: miR-101, miR-30A-3P, miR-200A, and miR-199A. The authors found 21 upregulated genes that were potential targets for at least three of the above-mentioned miRNAs, including oncogenes like *RUNX1/AML1* and *SS18*.

Other molecular events characterizing FTC and not related to its mutational status

The majority of the gene expression analyses of FTC (Barden *et al.* 2003, Cerutti *et al.* 2004, Finley *et al.* 2004a, Weber *et al.* 2005, Hinsch *et al.* 2009) have considered the diagnostic aspect and have searched for gene signatures or genes differentiating FTC from FA. Among the genes listed are cyclin D2, protein convertase 2 (*PCSK2*), and prostate differentiation factor (*PLAB*; Borup *et al.* 2010). Borup *et al.* performed a global expression profiling of follicular tumors and found expression changes in genes involved in DNA replication and mitosis, loss of growth arrest, and proapoptotic factors to be characteristic of FTC, among them being *NR4A1* and *A3*, encoding orphan nuclear receptors involved in the regulation of proliferation and apoptosis, as well as *FOSB* and *JUN*. Loss of apoptosis and growth arrest factors occurred during malignant transformation and was prominent in all samples, implying that this event preceded proliferation. The coordinated downregulation of the expression of *NR4A1* and *A3*, *JUN*, *FOSB*, and *CITED2* was a striking observation for these authors because it was contrary to their increased expression in other cancers. Interestingly, *NR4A1* and *A3* translocate to the mitochondria, where they stimulate the release of cytochrome C in a *BLC2*-dependent manner (Borup *et al.* 2010). The frequent loss of expression had been reported as a characteristic feature of FTC previously. The self-regulation of caveolin-1, both at the mRNA and protein levels, was one of the first expression changes

described in FTCs by Aldred *et al.* (2003), who also considered insular-type and oxyphilic (Hurthle-type) thyroid cancers.

Transcriptome of PDTC and undifferentiated thyroid cancer in relation to their mutational profile

The PDTC and undifferentiated anaplastic thyroid carcinomas (ATC) respectively present a much more aggressive clinical course than papillary or follicular thyroid cancers, which are frequently described as well-differentiated cancers (DTC). Their origin is not well defined but the prevalence of *BRAF*, *RAS* mutations, or genetic alterations of *PIK3CA* as well as the presence of pre-existing PTC areas observed in some PDTCs suggest that at least some of them may originate from DTCs (Garcia-Rostan *et al.* 2003, 2005, Nikiforova *et al.* 2003a). In addition, *RET/PTC* rearrangements were detected in PDTCs at a frequency of 10%, and the proportion increased to 20% when only PDTCs with areas of well-differentiated thyroid carcinoma were considered (Santoro *et al.* 2002). Although a mouse model of papillary thyroid carcinoma induced by *RET/PTC3* showed the formation of a solid PTC variant with metastases (Powell *et al.* 1998), there is no evidence of the association between *RET/PTC* and poor prognosis-associated PDTCs (Santoro *et al.* 2002). Wreesmann *et al.* (2002) noticed an increase in the incidence of DNA copy number changes from DTC to PDTC, both at the level of number of patients with abnormalities and number of abnormalities per case. Saltman *et al.* (2006) suggested an intermediate position for PDTC between the well-differentiated and anaplastic thyroid carcinomas, with the most significant changes represented by the Ki-67 protein, which was present in 5.8% of well-differentiated PTC, 48.8% of PDTCs, and 81.8% of ATCs. Nevertheless, the hypothesis that PDTC and ATC arise *de novo* cannot be excluded.

The deregulation of *TP53* (tumor protein p53) and Wnt pathways and mutations of β -catenin are common in both PDTC and ATC (Donghi *et al.* 1993, Garcia-Rostan *et al.* 1999). Lavra *et al.* (2009) showed a positive correlation between galectin-3 expression and *p53* mutations. Gal-3, an adhesion-related and simultaneously anti-apoptotic molecule, was upregulated in most ATCs and thyroid carcinoma cell lines harboring the most frequently detected *p53* mutation, R273H. Over-expression of the p53 protein (TP53) was also found to be correlated with the upregulation of its antagonist p63, a member of the p53 protein family (Malaguarnera *et al.* 2005) and the GLUT1 (Kim *et al.* 2006). Schwartzberg-Bar-Yoseph *et al.* (2004) demonstrated that *GLUT1* and *GLUT4* are repressed by wild-type p53, and mutations within the DNA-binding

domain of TP53 disable this interaction. These data help to explain the findings of Kim *et al.* (2006) showing over-expression of *TP53* and *GLUT1* in ATC. Guida *et al.* suggested dependency between mutated *TP53* and upregulation of minichromosome maintenance protein 7 (MCM7), a protein required for DNA replication, which was reported to be highly over-expressed in ATC but absent in normal thyroid and PTC (Guida *et al.* 2005).

Salvatore *et al.* (2007) presented a gene signature of anaplastic thyroid carcinoma characterized by the upregulation of genes involved in the regulation of cell cycle progression and chromosome segregation. The authors demonstrated that the Polo-like kinase 1 (PLK1), previously included in the 'proliferation cluster' and 'chromosomal instability 70 cluster' (Tabach *et al.* 2005, Carter *et al.* 2006, Whitfield *et al.* 2006), was required for ATC cell proliferation and cell survival, and this dependence was not detected in normal thyroid cells, in agreement with the data of Nappi *et al.* (2009). Pita *et al.* (2009) found a number of upregulated cell cycle-related genes in PDTC. Among them were genes associated with mitosis such as CDC28 protein kinase regulatory subunit 2 (*CKS2*) and cyclin E2 (*CCNE2*), which were previously reported as upregulated in many other types of tumors (Gudas *et al.* 1999, Scrideli *et al.* 2008). The authors stressed the over-expression of the ubiquitin-like, containing PHD and ring finger domains (*UHRF1*) gene in PDTC. This gene encodes a protein involved in processes of breaking and rejoining of DNA strands through the regulation of the TOP2A enzyme and is involved in the DNA damage response and, through the regulation of methyltransferase 1 (*DNMT1*), in VEGF regulation. The *UHRF1* gene is located within the chromosome region 19p13, in which Lee *et al.* (2008) by CGH-array reported gains in 67% of ATCs. Microarray analysis also showed a strong upregulation of *PKB* (v-AKT murine thymoma viral oncogene homolog 1), which encodes a serine/threonine kinase of the MAPK pathway, in PDTC. The over-expression of *PKB* in poorly differentiated thyroid carcinomas was first demonstrated by Rodrigues *et al.* (2007b), who emphasized the importance of the MAP kinase pathway in the progression of thyroid carcinomas. Montero-Conde *et al.* (2008) investigated differentiated and undifferentiated thyroid tumors by using cDNA microarrays and also reported significant changes in the expression of genes of the MAPK pathway, as well as those of the TGF β pathway, focal adhesion, and activation of actin polymerization and cell cycle.

Ito *et al.* (2009) reported the co-expression of S100A8 and A100A9, which represent the S100 calcium-binding proteins A8 and A9, in undifferentiated thyroid carcinomas, and the lack of *S100A8* expression and a weak expression of *S100A9* in PDTC, and lack of these proteins in well-differentiated DTC. Similar gradation was reported

by Aratake *et al.* (2010) who examined the expression of extracellular matrix metalloproteinase inducer (EMMPRIN) and found a weak expression level in well-differentiated DTC (4-9) and high levels in ATC (245-7).

With regard to genes downregulated (lost) in ATC, Rodrigues *et al.* (2007b) reported the downregulation of pleomorphic adenoma gene-like 1 (*PLAGL1*) and described under-expression of *CDH1* (cadherin 1, type 1, E-cadherin (epithelial)) in PDTC. Most downregulated genes described by Pita *et al.* in PDTC in comparison to normal thyroid tissue were related to cell adhesion. Deregulation of cell adhesion genes may promote tumor invasion and metastasis. However, there were only two genes differentially expressed when PDTC was compared to DTC, and 3-phosphoinositide-dependent protein kinase-1 gene (*PDPK1*) was the most interesting gene with regard to its function in AKT activation. Downregulation of the epithelial cell adhesion molecule (EpCAM) was reported by Ensinger *et al.* (2006), who reported its absence from undifferentiated thyroid carcinomas and high level of EpCAM expression in well-differentiated DTC and PDTC. Previous studies indicated the dual role of EpCAM, suggesting its association with tumor formation and oncogene characteristics (Spizzo *et al.* 2004). However, another study also described EpCAM as a tumor suppressive protein (Gosens *et al.* 2007). Because of the adhesive properties of EpCAM (Litvinov *et al.* 1994), a role of this protein in the inhibition of invasion was proposed.

Espadinha *et al.* (2009a) reported an association between the downregulation of PPAR γ and lower differentiation of thyroid follicular cells. These authors focused on the effect of PPAR γ on the function of E2F/DP, which regulates the expression of genes involved in entry into S phase and DNA synthesis and whose decrease was observed during the differentiation of several types of cells (La Thangue and Rigby 1987, Hara *et al.* 1993, Melamed *et al.* 1993).

With reference to miRNA expression in undifferentiated thyroid cancers, Visone *et al.* (2007) suggested that the deregulation of miRNA might be an important step in thyroid carcinogenesis based on the detection of a decrease in miR-30d, miR-125b, miR-26a, and miR-30a-5p in ATC compared with normal thyroid tissue. Not unexpectedly, the array-based analyses showed a marked under-expression of thyroid-specific genes in anaplastic thyroid carcinoma (Onda *et al.* 2004, Rodrigues *et al.* 2007b, Matsumoto *et al.* 2008).

Final remarks

The relationship between the presence of thyroid cancer histotype-specific mutations and the gene expression profiles of tumors is complex, and many factors related to cancer invasiveness and host

responses have to be considered. However, studies on the association of gene signatures with mutational events associated with carcinogenesis may help to elucidate the molecular consequences of the activation of oncogenes and the loss of tumor suppressors and thus improve our understanding of tumor biology and facilitate the search for prognostic and predictive markers and new therapy targets. The gene expression profile differences between papillary thyroid cancer and FTC and PDTC and undifferentiated thyroid cancer mainly reflect the differences in the degree of tumor differentiation. However, the observed profound differences between BRAF-positive and BRAF-negative PTCs, much more distinct than the respective differences in the gene expression signatures of FTCs with different mutations, are indicative of profound changes in biology related to the appearance of this particular oncogene.

Declaration of interest

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

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