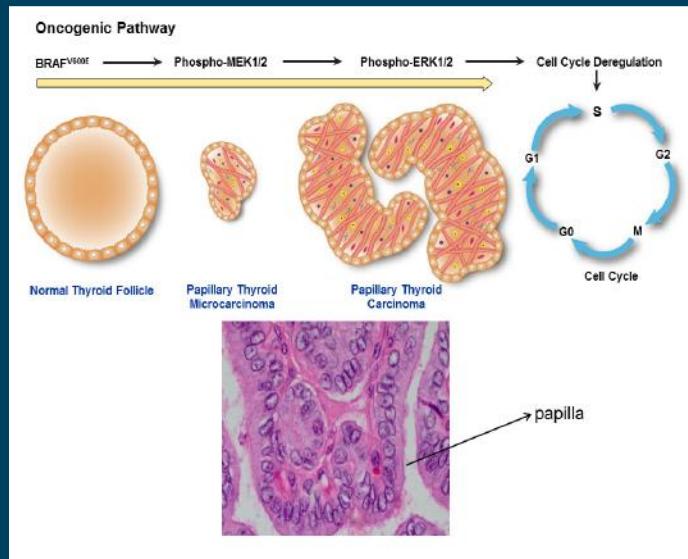


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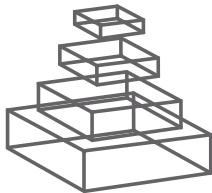


TARGETING THYROID CANCER MICROENVIRONMENT AND EPIGENETIC SIGNALLING: NEW FRONTIERS IN CANCER ENDOCRINOLOGY BASIC AND CLINICAL RESEARCH

Topic Editor
Carmelo Nucera



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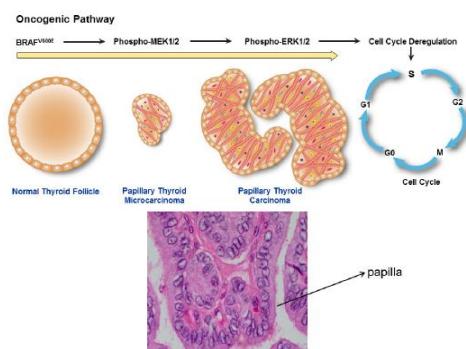
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TARGETING THYROID CANCER MICROENVIRONMENT AND EPIGENETIC SIGNALLING: NEW FRONTIERS IN CANCER ENDOCRINOLOGY BASIC AND CLINICAL RESEARCH

Topic Editor:

Carmelo Nucera, Beth Israel Deaconess Medical Center/Harvard Medical School, USA



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Scopes:

The scope of this Research Topic was to cover the entire field of thyroid cancers: the main focus of this topic is translational, with an emphasis on bench to bedside research.

Experimental, pre-clinical and clinical research addressing the following aspects is included in this Research Topic:

This Research Topic is devoted to the understanding of molecular mechanisms of Human Thyroid Cancers.

Original research describing functional studies of genetic mutations that shed novel insights into the aetiology and pathogenesis of these cancers, as well as angiogenesis and tumor microenvironment, mouse models studies that describe mechanisms or novel potential therapeutic targets and biomarkers for these endocrine cancers are presented.

- 1) Investigation of specific molecular patterns of thyroid tumorigenesis, which could allow the development of new directions in the field of pharmacotherapy research;
- 2) Emphasis on animal studies (preclinical models of human anaplastic thyroid cancers) for the validation of biomarkers with the potential to lead to clinical trials, and studies of targetable mechanisms of oncogenesis, progression of these malignancies, tumor microenvironment and extracellular matrix, and metastatic disease;
- 3) Assessment of biomarkers to predict the potential response or resistance to drug treatment (targeted cancer therapies) or to guide the follow-up of treated patients;
- 4) Investigation of new laboratory molecular tests (e.g. molecular techniques and applications of thyroid fine-needle aspiration biopsy) to translate in the clinical practice;

In summary, specific areas of interest include:

Thyroid cancer genetics; genome-wide analysis; clinical and translational research; orthotopic mouse models of metastatic thyroid carcinoma; tumor microenvironment; epigenetic; biological insights of personalized medicine; novel applications of bioinformatics; large scale molecular characterization of tumors; diagnostic or prognostic biomarkers; endocrine pathology studies; thyroid fine-needle aspiration.

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Targeting thyroid cancer microenvironment: basic research and clinical applications

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Keywords: thyroid cancer, microenvironment, BRAF^{V600E}, translational, targeted therapy, biomarkers, extracellular matrix, mouse models

INTRODUCTION

Thyroid cancer is the most common endocrine malignancy and its incidence has increased considerably over the past few decades (1). This Research Topic is devoted to understanding the molecular mechanism of human thyroid cancer, with an emphasis on translation to the clinic. The original research papers assembled here probe the pathogenetic pathway from a wide range of approaches, including: (1) the role of microenvironment in thyroid cancer, (2) the role of deiodinases and epigenes leading to thyroid cancer, (3) functional studies of genetic mutations that shed insights into the etiology and pathogenesis of papillary thyroid micro-carcinoma or carcinoma (PTC), (4) genome-wide studies that describe patho-physiological mechanisms for thyroid cancer, and (5) epidemiological studies of thyroid cancer incidence and (6) the influence of environmental factors on development of human PTC.

SCOPE

The articles gathered here reflect a particular interest in the genetic and epigenetic factors that could affect the thyroid cancer cell and its microenvironment. In particular, this topic will present pre-clinical and clinical research addressing the following issues: (i) identification of specific molecular patterns of thyroid tumorigenesis and tumor microenvironment, which could suggest new directions in pharmacotherapy research; (ii) discovery of new biomarkers to predict response or resistance to drug treatment, facilitating targeted cancer therapies and patient follow-up after treatment; (iii) discovery of environmental risk factors that might affect PTC development; and (iv) evaluation of preclinical models of human thyroid cancers with regard to their suitability for testing new drugs and molecule-targeted agents and for studies of targetable mechanisms of oncogenesis, malignant progression, and metastatic disease.

Specific areas of investigation in this topic include the following as they relate to thyroid cancer: epidemiology and clinical research; epigenetics in thyroid; personalized medicine in thyroid; the tumor microenvironment; novel applications of bioinformatics in human thyroid cancer cell lines; molecular characterization of thyroid tumors; and use of diagnostic and prognostic biomarkers.

WHY IS TARGETING THE THYROID CARCINOMA MICROENVIRONMENT TRANSLATIONAL?

Currently no successful treatment is available for advanced thyroid cancer, including poorly differentiated (PDTC), anaplastic/undifferentiated (ATC), and metastatic recurrent/persistent differentiated PTC that is not responsive to radio-iodine therapy.

The past decade of thyroid cancer research has yielded fundamental advances with profound translational potential. Identification of molecular markers, including oncogenes (e.g., the BRAF^{V600E} mutation), tumor suppressors (e.g., TP53 mutations), and translocations (e.g., RET/PTC, PAX8/PPAR γ), have clarified molecular mechanisms underlying the pathogenesis of thyroid cancer. However, correlations between each biomarker and prognosis/outcome have not yet been determined in a broad cohort of patients with metastatic thyroid cancer. Currently, BRAF^{V600E} is the most frequent genetic hallmark of PTC and has been highlighted as a prognostic biomarker to improve risk stratification of patients with PTC, including low risk PTC (2–7). Recently, a retrospective multicenter study has also shown that the occurrence of the BRAF^{V600E} mutation was significantly associated with increased cancer-related mortality among patients with PTC. However, because mortality in patients with PTC was low and the association was not independent of PTC clinico-pathologic features, the role of BRAF^{V600E} as marker of mortality risk in patients with PTC remains to be determined (3).

Preclinical studies have shown that PTC carrying the BRAF^{V600E} mutation are dependent on this oncoprotein for viability; both genetic and pharmacological inhibition of BRAF^{V600E} expression or activity is associated with thyroid carcinoma regression and restoration of radio-iodine uptake *in vivo* in mice (8). Furthermore, BRAF^{V600E} plays an important role in PTC progression to ATC through genes fundamental in the regulation of tumor microenvironment by triggering tumor invasion and metastasis (9). Therefore, testing a patient's thyroid cancer for BRAF^{V600E} will yield important information about potential tumor aggressiveness and inform future use of targeted therapies with selective BRAF^{V600E} inhibitors. Collectively, these findings suggest a potential translational application for anti-BRAF^{V600E}

therapy in clinical trials for patients with thyroid cancers refractory to radio-iodine treatment and surgically inoperable thyroid cancers.

BRAF^{V600E} affects the expression of tumor extracellular matrix (ECM) non-cellular components [e.g., Thrombospondin-1 (TSP-1), integrins and others] (9) by regulating PTC cell microenvironment communications; indeed, the molecular action of *BRAF*^{V600E} appears to affect both the migratory and invasive properties of the human thyroid cancer cell itself (10), as well as other cell types of the thyroid tumor microenvironment. Knowledge about new *BRAF*^{V600E}-dependent targets (11) may help identify secreted factors that could serve as novel prognostic biomarkers and/or innovative therapeutic strategies in *BRAF*^{V600E}-positive human thyroid cancers. For example, tumor-associated lymphocytes and high FoxP3(+) regulatory T cell (Treg) frequency in primary PTC correlates with more aggressive disease (12, 13). This suggests Treg frequency could be a predictive factor in PTC and that the suppressive effects of Treg could be considered in the design of immune-based therapies in PTC. Also, Ryder et al. found that tumor-associated macrophages (TAMs) may facilitate thyroid cancer progression (14), showing that the presence of a high density of TAMs in advanced metastatic thyroid cancers correlates with invasion and decreased cancer-related survival.

In summary, novel therapeutic strategies that target the metastatic thyroid carcinoma microenvironment (i.e., ECM cellular and non-cellular components) could offer an additional approach to the treatment of patients with these types of cancers. Targeting other cell types in the microenvironment instead of, or in addition to, the *BRAF*^{V600E}-positive metastatic thyroid cancer cell might also minimize anti-*BRAF*^{V600E} drug resistance and provide potential additional therapeutic benefits. Determining the effects of factors in the thyroid tumor microenvironment will further define the spectrum of molecular mechanisms underlying signaling in metastatic thyroid carcinomas. Understanding the extent to which microenvironment factors participate in the aberrant behavior of *BRAF*^{V600E}-positive human metastatic thyroid cancer cells will reveal whether the microenvironment is a promising target for development of new therapies. In summary, current research suggests that novel therapies directed against “microenvironment-specific targets” of thyroid carcinoma are a promising approach and should be developed and tested in pre-clinical/translational models of human metastatic thyroid cancer in the near future.

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Thrombospondin-1 silencing down-regulates integrin expression levels in human anaplastic thyroid cancer cells with BRAF^{V600E}: new insights in the host tissue adaptation and homeostasis of tumor microenvironment

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Background and Rationale: Anaplastic thyroid cancer (ATC) is characterized by pleomorphic cells, has a poor prognosis, is highly devastating disease, and is not curable. No reliable biomarkers of metastatic potential, helpful for early diagnosis of ATC and therapeutic response have been found yet. Thrombospondin-1 (TSP-1) plays a fundamental role in cancer progression by regulating cell stromal cross-talk in the tumor microenvironment.

Goals: Our goal was to understand whether TSP-1 could affect protein levels of its integrin receptors (e.g., ITG α 3, α 6, and β 1) and cell morphology in BRAF^{V600E}-ATC cells *in vitro* and *in vivo*.

Experimental Design: Anaplastic thyroid cancer-derived cell cultures and western blotting were used to assess integrin protein expression upon TSP-1 silencing. Immunohistochemistry was performed on orthotopic primary human ATC and metastatic ATC in lung tissue to compare TSP-1 and integrin protein expression levels.

Results: TSP-1 knock-down down-regulates ITG α 3, α 6, and β 1 in BRAF^{V600E}-human ATC cells. BRAF^{V600E}-ATC cells with TSP-1 knock-down were rounded compared to control cells, which displayed a spread morphology. TSP-1 knock-down also reduced TSP-1, ITG α 3, α 6, and β 1 protein expression levels *in vivo* in the ATC microenvironment, which is enriched in stromal and inflammatory cells.

Conclusion: TSP-1 silencing causes changes in ITG levels and ATC cell morphology. The assessment of TSP-1 and ITG levels might contribute to earlier metastatic potential of BRAF^{V600E}-positive aggressive thyroid cancers, and allow improved patient selection for clinical trials.

Keywords: BRAF^{V600E}, integrins, thyroid cancer, microenvironment, extracellular matrix, TSP-1

INTRODUCTION

The incidence of thyroid cancer is increasing more rapidly than other cancers in the US (1) and in other countries (2). Anaplastic thyroid cancer (ATC) has perhaps the worst prognosis of any cancer, with a median survival of about 5 months and a 20% 1-year survival rate (3). ATC is resistant to standard chemotherapy, external beam radiation, and radioiodine treatment (3), thus new treatments are urgently needed. Outcomes could be improved with routine assessment of pro-metastatic biomarkers, which could enable earlier metastatic potential of this type of fatal thyroid cancer. The BRAF^{V600E} mutation is the most prevalent genetic alteration (greater than 50%) in papillary thyroid cancer (PTC) and is implicated in the progression of PTC to ATC (4–6), a crucial challenge in thyroid cancer. Our previous studies demonstrated

the pro-metastatic role of the secreted extracellular matrix (ECM) protein thrombospondin-1 (TSP-1) in BRAF^{V600E}-positive PTC (5, 7, 8) and indicated that TSP-1 increased phosphorylation of ERK1/2 (5). Gene Set Enrichment Analysis (GSEA) (5) was performed on a cohort of BRAF^{V600E} or BRAF^{WT} PTC specimens and normal thyroid tissue (NT) samples. We found 18 independent gene sets (of 539 tested) significantly associated with BRAF^{V600E} PTCs: 17 up-regulated and 1 down-regulated set (5). The GSEA data revealed that TSP-1 and several integrins were up-regulated in the BRAF^{V600E}-positive human PTC (5).

TSP-1 binds to a wide variety of integrins, however the best characterized are integrin alpha3/beta1 and alpha6/beta1 (ITG α 3/ITG β 1 or ITG α 6/ITG β 1) (9–11). TSP-1 also binds non-integrin cell surface receptors (i.e., proteoglycans, CD36, CD47),

matrix proteins [i.e., Fibronectin (FN)], cytokines (i.e., TGF- β 1), pro-angiogenic factors (e.g., VEGF), and matrix proteases (i.e., MMP-9), indicating its importance in cross-talk between ECM molecules and their receptors (11, 12). Also, TSP-1 is involved in tumor cell adhesion and migration, and it may direct clustering of receptors to specialized domains for these biological processes (10). Integrins are a family of cell surface glycoproteins that function as receptors for ECM proteins, mediating both cell–cell and cell–ECM adhesion. Integrins are non-covalent, heterodimeric complexes of an alpha (α) and a beta (β) subunit (13). Their role is fundamental in cell microenvironment homeostasis, including either physiological or pathological conditions. Whereas, the role of TSP-1 in angiogenesis is well documented, its role in tumor metastasis is only just emerging. TSP-1 has been shown to promote metastasis in a breast cancer model (14). Our previous study has shown that the N-terminal domain of TSP-1 is involved in BRAF^{V600E}-mediated invasion in thyroid cancer cells (5). Chandrasekaran et al. (10) also showed a critical role for the TSP-1 N-terminal domain in breast cancer cell invasion via putative binding site(s) to ITG α 3/ITG β 1, which has an important role in tumor cell migration and invasion. Sumimoto et al. (15) have shown that BRAF^{V600E} knock-down decreased phospho-ERK1/2 protein levels and inhibited invasion of melanoma cells accompanied by a decrease of matrix metalloproteinase activity and ITG β 1 expression. Dynamic and reciprocal interactions involving cell adhesion molecules (e.g., integrins, CD44), ECM non-cellular components (i.e., TSP-1, FN), and soluble cytokines occur between tumor epithelial cells and tumor microenvironment stromal cells (13). Importantly, TSP-1 could be a valid biomarker for PTC aggressiveness and we have already established an immunohistochemistry (IHC)-based screening assay suitable for clinical trials (5). The goal of this brief research article is to understand whether TSP-1 affects integrin levels and cell morphology in BRAF^{V600E}-positive ATC cells, contributing to metastasis.

MATERIALS AND METHODS

CELL CULTURE

The SW1736 ATC cell line, which harbors heterozygous BRAF^{WT/V600E}, was kindly provided by N. E. Hedin (University of Uppsala, Uppsala, Sweden). The 8505c ATC cell line homozygous for BRAF^{V600E/V600E} was purchased from DSMZ (German collection of microorganisms and cell culture) (Brunswick, Germany). The 8505c cell line was established by Dr. M. Akiyama (Radiation Effects Research Foundation, Hiroshima, Japan) from the primary tumor of a 78-year-old woman with undifferentiated carcinoma. It is histologically an ATC with some spindle, polygonal, and giant cells (data by DSMZ). SW1736 and 8505c cell lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin/amphotericin.

ANTIBODIES

Antibodies against the following proteins were used: β -actin (A-5316) (Sigma); TSP-1 (A6.1) for IHC (Abcam, USA), and the previously validated TSP-1 clone MA-I for Western blot (5, 16) and R1 for immunofluorescence (17, 18); anti-ITG α 3 (C-18, Santa Cruz Biotechnology, USA), anti-ITG α 6 (H-87, Santa Cruz Biotechnology, USA), and anti-ITG β 1 (kindly provided from Dr. Richard Hynes, MIT, Cambridge, MA, USA); anti phospho-FAK (cat.

#3283, Cell Signaling, USA), and anti-total FAK (cat #3285, Cell Signaling, USA). CD45 (cat#550539, BD Pharmingen, USA), F4-80 (cat# 14-4801, eBioscience, USA), and α SMA (alpha-smooth muscle actin) (A2547, Sigma, USA).

CELL TRANSFCTIONS FOR LENTIVIRUS PRODUCTION

HEK 293T cells (5×10^5) were grown in 60-mm plates and transfected using Fugene-6 (Roche) in OptiMEM (Invitrogen) for 48 h according to the manufacturer's instructions.

TSP-1 SILENCING TECHNIQUES

Stable transduced [shRNA (sh) viral transductions] ATC cells with or without TSP-1 knock-down were established according to Nucera et al. (5).

WESTERN BLOTTING

Western blotting assays were performed following standard protocols; cells were lysed in buffer, composed of 10 mM Hepes (pH 7.40), 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 5 mM sodium fluoride, and 1% Triton-X 100; protease and phosphatase inhibitors (Pierce) were used for protein extractions (5).

IN VIVO STUDIES

The animal work was done in the animal facility at Beth Israel Deaconess Medical Center (Boston, MA, USA) in accordance with federal, local, and institutional guidelines. We used an orthotopic mouse model of ATC as previously described and validated by Nucera et al. (19) (5), using female about 6-week-old severe combined immunodeficient (SCID) mice (Taconic, USA).

HISTOLOGY AND IMMUNOHISTOCHEMISTRY

All tissue specimens (five primary orthotopic ATC or lung specimens from mice with sh-control TSP-1 8505c cells; and five primary orthotopic ATC or lung specimens from mice with sh-TSP-1 knock-down 8505 cells) were fixed with 10% buffered formalin phosphate and embedded in paraffin blocks. Histopathology evaluation was performed by a pathologist (Peter M. Sadow) on hematoxylin and eosin-stained tissue sections of orthotopic 8505c ATC specimens (5). All photos were captured with an Olympus BX41 microscope and the Olympus Q COLOR 5 photo camera (Olympus Corp., Lake Success, NY, USA), using the Twain software in Adobe Photoshop (7.0) and white balanced with the same method for all images. Sections (4 μ m thick) of formalin-fixed orthotopic 8505c ATC specimens (5) were used for IHC procedures. After baking overnight at 37°C, deparaffinization with xylene/ethanol and rehydration were performed. IHC analysis was performed using primary antibodies against human TSP-1 (1:25, citrate buffer for antigen retrieval), ITG α 3 (1:250, citrate buffer for antigen retrieval), ITG α 6 (1:200, citrate buffer for antigen retrieval), or ITG β 1 (1:500, citrate buffer for antigen retrieval); anti-mouse CD45 antibody (#550539, BD Pharmingen, USA): 1:50, citrate buffer and pressure cooker for antigen retrieval; anti-mouse F4-80 antibody (pan macrophage marker) (#144801, eBioscience, USA): 1:50, citrate buffer and pressure cooker for antigen retrieval; and anti-mouse alpha-smooth muscle actin (α SMA) (#A-2547, Sigma, USA) (1:20,000). The sections, treated with pressure cooker for antigen retrieval (Biocare Medical, Concord,

CA, USA), were incubated at 123°C in citrate buffer (Dako Target Retrieval Solution, S1699; DAKO Corp.), cooled and washed with PBS. Antigen retrieval was performed for 60 min at room temperature. The primary antibody was detected using a biotin-free secondary antibody (K4011) (Dako Envision system) and incubated for 30 min. All incubations were carried out in a humid chamber at room temperature. Slides were rinsed with PBS between incubations. Sections were developed using 3,3-diaminobenzidine (Sigma Chemical Co.) as a substrate and were counterstained with Mayer's hematoxylin. PAX8 and p53 immunostaining was performed according to our previous study (19). We used species-specific IgG as negative control. The IHC markers were assessed semiquantitatively using the following scoring method: 0 negative, 1 1–10% positive cells (low expression), 2 11–50% positive cells (moderate), and 3 more than 50% positive cells (high expression) according to Shaik et al. (20).

IMMUNOFLUORESCENCE

For immunofluorescence experiments, 5×10^4 8505c or SW1736 cells were seeded on type I collagen-coated cover slips (BD Biosciences) for 24 h. Cells were washed three times with PBS, fixed with 4% paraformaldehyde for 10 min at room temperature, and permeabilized with PBS 0.5% Triton-X 100 for 5 min at room temperature. After three washes with PBS, cells were blocked with TBST 1% BSA for 20 min, followed by incubation with phalloidin-fluorescein (Sigma, USA) in PBST 1% BSA for 30 min

at room temperature. Cells were rinsed three times with TBS. Finally, the cover slips were mounted with a mixture of Vectashield mounting medium and DAPI (Vector Laboratories). Cells were imaged at 20 \times on a Nikon Eclipse 300 epifluorescence inverted microscope connected to a Retiga 2000RV camera (Nikon Instruments).

STATISTICAL ANALYSIS

Statistical analysis was carried out using Microsoft Excel Software. Results were compared using the Student's *t*-test and χ^2 test. *P* values of <0.05 were considered significant ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$). Densitometry analysis was performed using Quantity One software (BioRad, USA).

RESULTS

KNOCK-DOWN OF TSP-1 DOWN-REGULATES INTEGRINS LEVELS AND AFFECTS CELL MORPHOLOGY IN HUMAN ANAPLASTIC THYROID CANCER CELLS WITH BRAF^{V600E}

Our results show that ITG α 3 (~95%), ITG α 6 (~95%), and ITG β 1 (~90%) subunits are decreased in homozygous BRAF^{V600E}-positive 8505c ATC cells with TSP-1 knock-down (Figure 1). Furthermore, either homozygous BRAF^{V600E}-positive 8505c ATC cells or heterozygous BRAF^{V600E}-positive SW1736 ATC cells with TSP-1 knock-down display a markedly different morphology (rounded cells) compared to sh-controls cells (spread morphology) when plated on type 1 collagen (Figure 2). We also found

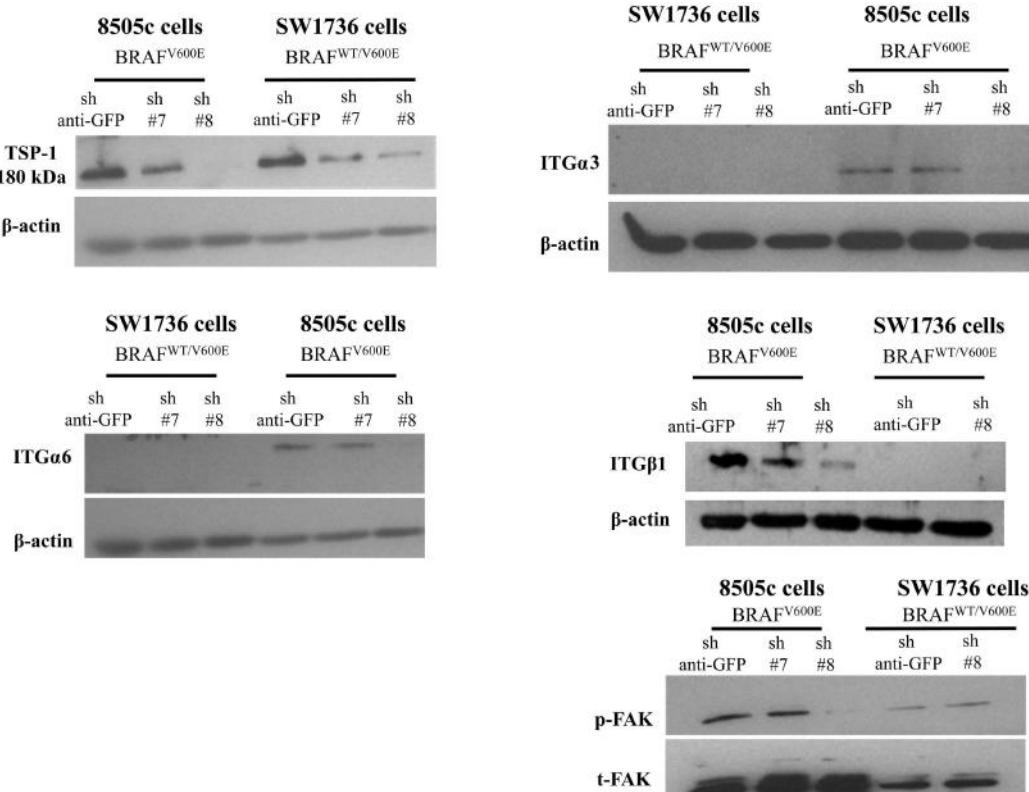
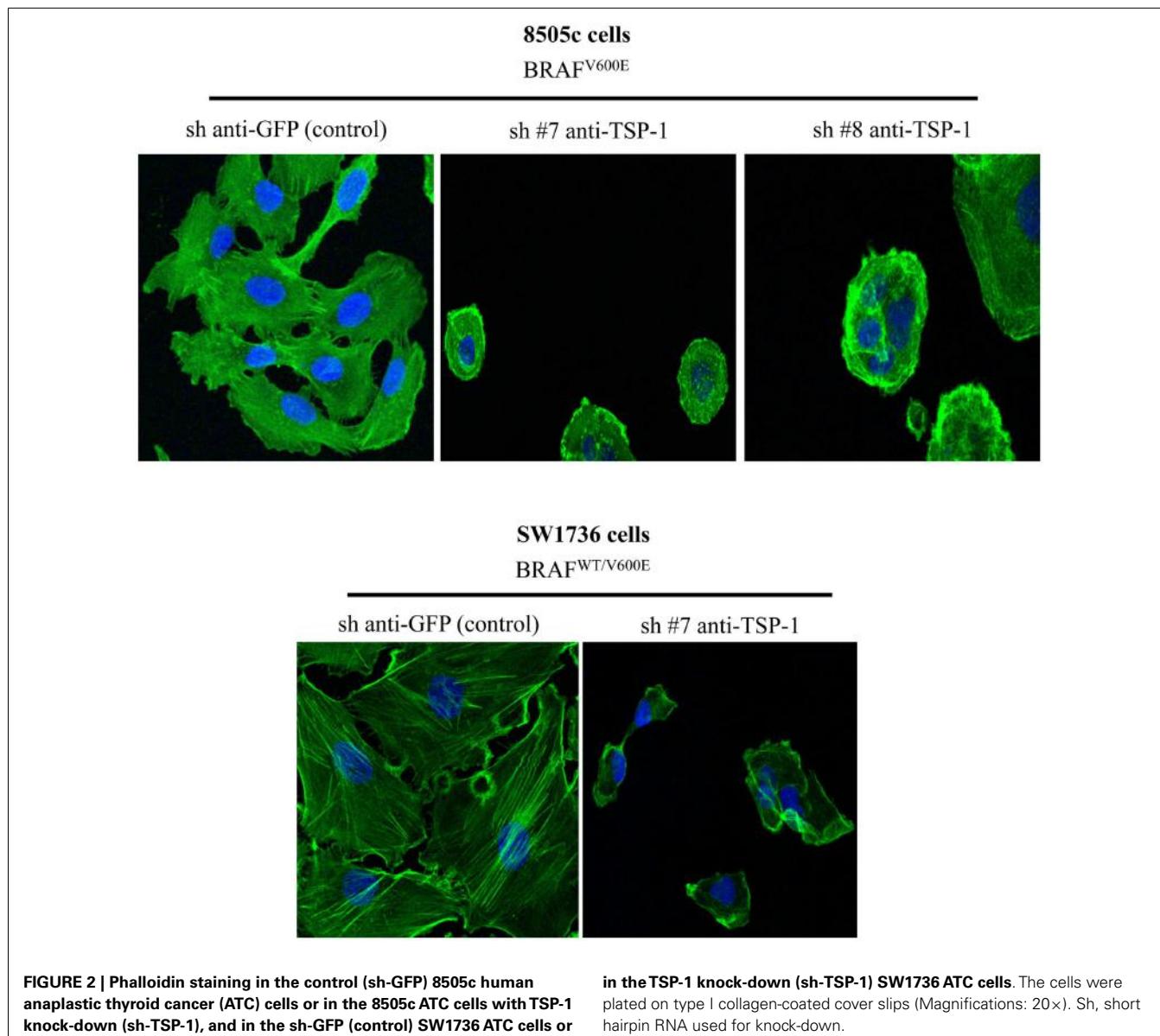


FIGURE 1 | ITG α 3, ITG α 6, and ITG β 1 protein levels by western blot in human anaplastic thyroid cancer (ATC) cells with sh-GFP (green fluorescent protein, control) or knock-down of TSP-1 (#7 and 8) harboring heterozygous BRAF^{WT/V600E} (SW1736) or homozygous BRAF^{V600E} (8505c cells).



that TSP-1 knock-down by shRNA (sh) caused a down-regulation of pFAK protein levels (~90%) in human thyroid cancer cells with homozygous BRAF^{V600E} (Figure 1).

TSP-1 KNOCK-DOWN DOWN-REGULATES INTEGRINS LEVELS IN THE ORTHOTOPIC BRAF^{V600E} METASTATIC 8505 ATC CELLS *IN VIVO*

The orthotopic human BRAF^{V600E}-positive 8505c ATC microenvironment *in vivo* shows stromal cells, identified by expression of some marker proteins: CD45+ (lymphocytic lineage), F4/80+ (macrophages) (21, 22), and αSMA+ (spindle-shaped pericytes) (23) (Figure 3).

The 8505c cells with TSP-1 knock-down metastasize less (5) and show very low protein levels of (i) ITGα3 [from 10 ITGα3-positive cells (score 2) in the sh-control 8505 orthotopic tumors to 2 cells ± 0.5/field in the sh-TSP-1 8505c orthotopic tumors, score: 1+, $P < 0.05$]; (ii) ITGα6 [from 16 ITGα6-positive cells (score 2)

in the sh-control 8505 orthotopic tumors to 3 cells ± 0.35/field; score: 1+, $P < 0.05$]; and (iii) ITGβ1 [from 12 ITGβ1-positive cells (score 2) in the sh-control 8505 orthotopic tumors to 2 cells ± 0.42/field; score: 1, $P < 0.05$] in the primary orthotopic 8505c ATC (Figures 4A,B), as well as in the lungs (Figure 4C) where generally 8505c ATC cells preferentially metastasize (19). IHC staining in the TSP-1 knock-down condition (Figure 4C) highlights TSP-1 and integrin protein expression in macrophages (noted by the dot-like, granular cytoplasmic staining, and bland histomorphology) but not in the metastatic 8505c cells. Additionally, lung tissue from the 8505c ATC orthotopic mice with TSP-1 knock-down was also completely negative for both PAX8 and p53 protein expression that show prominent nuclear staining in 8505c cells which were absent (data not shown) (19). Furthermore, we have also found that TSP-1 [3 cells positive/field (score 2)], ITGα3 [6 cells positive/field (score 2)], and ITGβ1 [2 cells positive/field

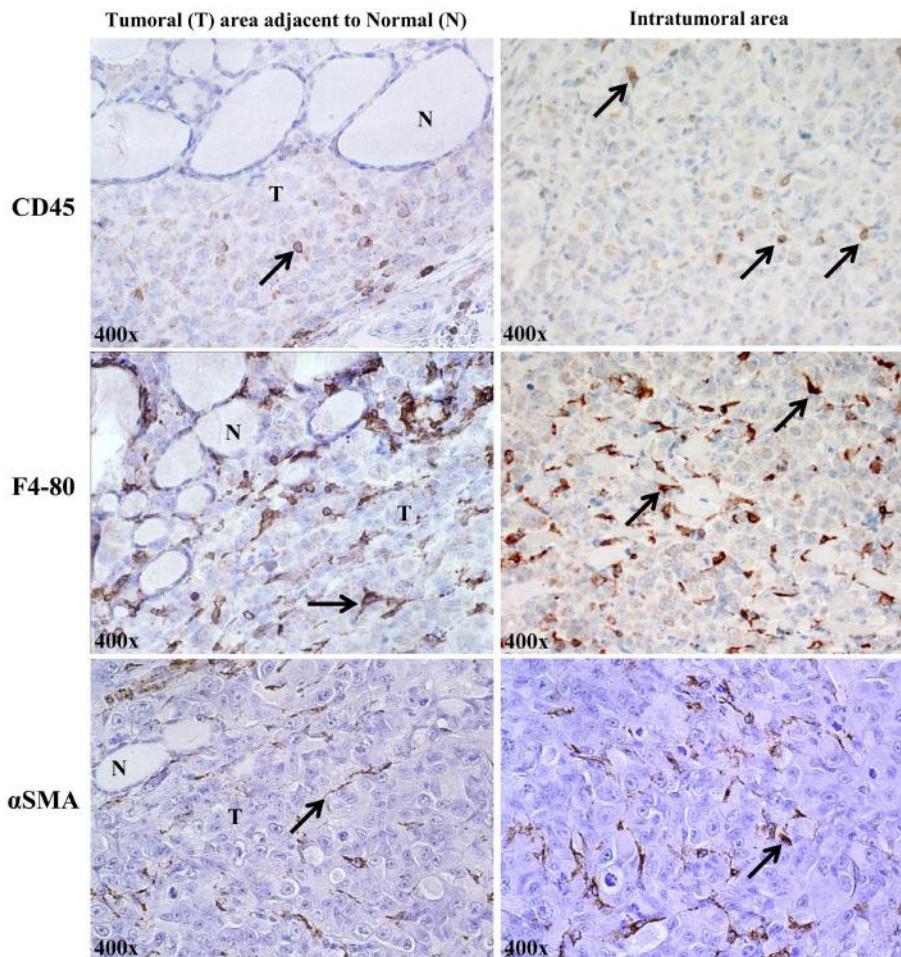


FIGURE 3 | Stromal cells in the orthotopic 8505c human anaplastic thyroid cancer (ATC) microenvironment of SCID immunocompromised mice (400 \times magnification, representative of eight mice). About 2% of cells/field were of lymphocytic lineage (CD45, localized to plasma

membrane); ~8% were identified as macrophages [F4/80 (pan macrophage marker), localized to plasma membrane]; and ~5% were spindle-shaped pericytes, identified by staining for cytosolic α SMA (alpha-smooth muscle actin).

(score 1)] proteins were expressed in the metastatic 8505c cells in the lungs but not up-regulated compared to the their expression in the primary orthotopic 8505c human ATC cells (Figure 4), thus suggesting that the potential intravasation and colonization of ATC cells do not up-regulate the basal protein expression levels of TSP-1, ITG α 3, and ITG β 1, which might be sufficient to trigger metastasis. By contrast, we found that ITG α 6 protein expression levels [2 cells positive/field (score 2)] were up-regulated in the orthotopic metastatic 8505c ATC cells in the lungs compared to the primary orthotopic 8505c ATC cells in the mouse thyroid.

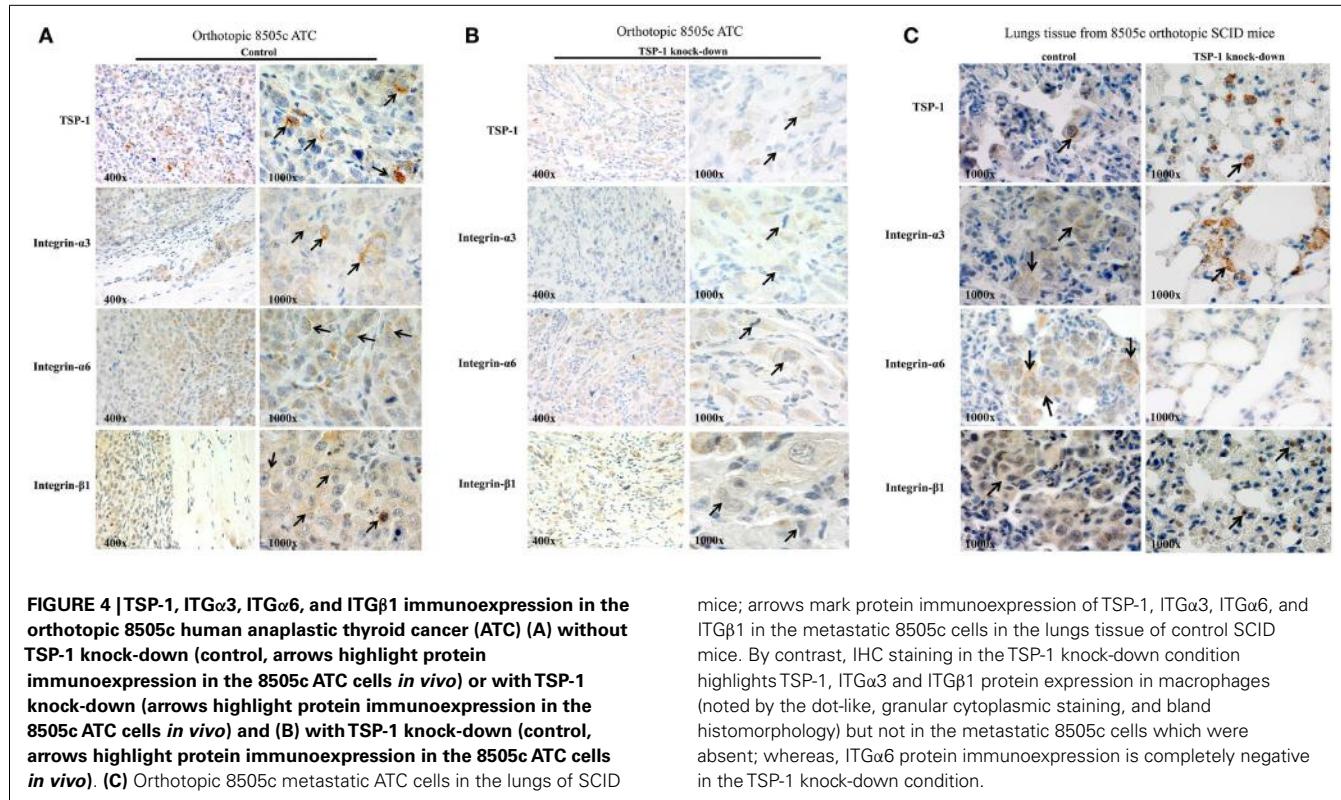
DISCUSSION

Thyroid carcinomas are increasing in frequency and account for 2.5% of all cancers in the United States (USA). ATC is a devastating disease with low survival rate due to uncontrolled systemic metastasis and with no effective treatment options. ATC represents 1.7% of all human thyroid cancers in USA, and geographically its prevalence ranges from 1.3 to 9.8% (median = 3.6%) (3). Conventional ATC therapy currently utilizes a multimodal

approach using radiation therapy and chemotherapeutic agents such as doxorubicin. The local disease burden often is so extensive that surgery is confined to tumor debulking and securing the airway. Additionally, no reliable biomarkers of metastatic potential, helpful for early diagnosis of ATC and therapeutic response have been found yet.

ATC shows a pleomorphic and interconnected network between high-grade malignant cells and stromal cells (e.g., macrophages) that characterize tumor microenvironment (24). Here our results from an *in vivo* orthotopic mouse model of human ATC also suggest that the ATC microenvironment is enriched in stromal cells, including macrophages, leukocytes, endothelial cells, and pericytes, which might contribute to the ATC aggressiveness.

Changes in the tumor microenvironment (e.g., deregulation of ECM molecules or ECM receptors) are a critical step in human cancer invasion, metastasis, and progression. We found that TSP-1 (a key player for ECM remodeling) and integrins (ECM receptors) are associated with BRAF^{V600E}-positive PTC



(5). We also showed that BRAF^{V600E} or TSP-1 knock-down (5) down-regulated ERK1/2 phosphorylation protein levels and inhibited ATC cell proliferation, migration, and invasion, all properties associated with integrin-mediated interactions with the ECM.

TSP-1 is a secreted/soluble protein that can be assessed in the plasma of patients with breast cancer, and shows significantly higher plasma concentrations than normal individuals or patients with benign breast disease (25, 26). It plays an important role in the physiology and pathology of the cell microenvironment (7, 12, 27, 28), and it has been proposed to have both pro-metastatic and anti-metastatic properties (14, 29). TSP-1 in the mammary tumor microenvironment inhibits angiogenesis and breast cancer growth, but promotes metastasis to the lung in a transgenic model of breast cancer (14). The ability of TSP-1 to support metastasis correlates with its ability to promote tumor cell migration (14).

Integrins are TSP-1 receptors that mediate tumor cell-ECM adhesion and provide both the connection to the adhesive substrate and cellular signaling crucial for cell proliferation, migration, and invasion (9, 13). To our best knowledge, this is the first report that shows that TSP-1 affects protein levels of integrins in BRAF^{V600E}-positive human ATC cells *in vitro* and *in vivo*. Also, TSP-1 knock-down *per se* significantly alters ATC cell morphology on type 1 collagen. Collectively, our results may suggest that BRAF^{V600E}-ATC cells might acquire an adaptation in the host tissue during their tissue colonization, reprogram their gene expression profile and up-regulate integrin (i.e., ITGα6) protein levels. This observation is supported from a recent report that shows that TSP-1 stimulates ITGα6 protein expression levels in human breast carcinoma cells, promoting tumor cell adhesion and invasion (9).

Additionally, the missense SNP rs11895564 (Ala380Thr) in ITGα6 may be a risk factor of thyroid cancer, contributing to the progression of PTC (30). Also, our results suggest that the zygosity (e.g., homozygous vs. heterozygous allelic mutations) of the BRAF^{V600E} mutation represents an important factor to take under consideration as a molecular modulator of the expression levels of integrins. In fact, the expression levels of many target genes could depend on the oncogenic dosage in human cancer cell. However, further studies are needed to understand better this aspect and the molecular role of integrins in thyroid cancer metastasis and progression.

Furthermore, integrins can also activate the FAK signaling cascade and promote PI3K kinase activity, which is essential to promote cancer invasion (31). Here, our results may suggest that TSP-1 protein levels also affect phospho-FAK protein levels, highlighting that TSP-1 might not only stimulate the ERK1/2 phosphorylation but additionally drive thyroid cancer cell adhesion and migration through FAK pathway(s). Shibue and Weinberg (31) demonstrated that ITGβ1 is fundamental to activate FAK signaling axis in controlling the initial proliferation of micro-metastatic mouse breast cancer cells disseminated in the lungs (31).

Overall, these results suggest that TSP-1 is a pro-metastatic constituent and TSP-1 silencing causes changes in integrin expression levels and ATC cell morphology. Therapeutic strategies aimed at modulating the thyroid cancer microenvironment might provide an additional perspective for the treatment of patients with these types of cancers. Routine assessment of pro-metastatic biomarkers, including TSP-1 and integrins, will help monitor patients undergoing targeted therapies, enable earlier metastatic potential of aggressive BRAF^{V600E}-positive human thyroid cancer, foster development of innovative therapies for refractory thyroid cancer

to current treatments, and allow improved patient selection for clinical trials.

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Association of cigarette smoking with aberrant methylation of the tumor suppressor gene *RARβ2* in papillary thyroid cancer

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Aberrant gene methylation is often seen in thyroid cancer, a common endocrine malignancy. Tobacco smoking has been shown to be associated with aberrant gene methylation in several cancers, but its relationship with gene methylation in thyroid cancer has not been examined. In the present study, we investigated the relationship between smoking of patients and aberrant methylation of tumor suppressor genes for TIMP3, SLC5A8, death-associated protein kinase, and retinoic acid receptor β 2 (*RARβ2*) in papillary thyroid cancer (PTC), the most common type of thyroid cancer. The promoter methylation status of these genes was analyzed using quantitative real-time methylation-specific PCR on bisulfite-treated genomic DNA isolated from tumor tissues and correlated with smoking history of the patients. Among the four genes, methylation of the *RARβ2* gene was significantly associated with smoking and other three genes showed a trend of association. Specifically, among the 138 patients investigated, 13/42 (31.0%) ever smokers vs. 10/96 (10.4%) never smokers harbored methylation of the *RARβ2* gene ($P = 0.003$). This association was highly significant also in the subset of conventional variant PTC ($P = 0.005$) and marginally significant in follicular variant PTC ($P = 0.06$). The results demonstrate that smoking-associated aberrant methylation of the *RARβ2* gene is a specific molecular event that may represent an important mechanism in thyroid tumorigenesis in smokers.

Keywords: thyroid cancer, tobacco smoking, methylation, tumor suppressor gene, *RARβ* gene

INTRODUCTION

Thyroid cancer is a common endocrine malignancy with a rapidly rising incidence in recent decades and at least 458,403 patients with thyroid cancer are currently living in the United States (Howlader et al., 2011). The most common histological type of this cancer is papillary thyroid cancer (PTC), which accounts for >80% of all thyroid malignancies (Hundahl et al., 1998; Howlader et al., 2011). PTC can be further divided into several subtypes, including mainly, in the order of decreasing prevalences, conventional, follicular variant and tall-cell PTC. Among the molecular derangements that drive thyroid tumorigenesis are epigenetic alterations, particularly aberrant gene methylation (Xing, 2007). Gene methylation is the covalent addition of a methyl group to the fifth carbon position of the cytosine residue in a CpG dinucleotide, which is usually associated with gene silencing and is a common mechanism for functional loss of key genes in human cancers (Esteller, 2008). Aberrant gene methylation in thyroid tumorigenesis often involves major tumor suppressor genes, such as those for the tissue inhibitor of metalloproteinase-3 (TIMP3), SLC5A8, death-associated protein kinase (DAPK), and retinoic acid receptor β 2 (*RARβ2*), particularly in PTC (Hoque et al., 2005; Hu et al., 2006). The tumor suppressor functions of these genes have been well characterized and they are frequently methylated in cancers. For example, the anti-tumor role of the *RARβ2* gene, which is often silenced by aberrant methylation in cancers, is mediated through

inhibition of cancer cell proliferation and metastasis and induction of apoptosis (Raffo et al., 2000; Widschwenter et al., 2001; Treuting et al., 2002). Conversely, re-expression of *RARβ2* is associated with significant reduction of cell growth in thyroid cancer cells (Miasaki et al., 2008).

Few environmental risk factors for thyroid cancer are known. Ionizing radiation exposure, the best-established risk factor for thyroid cancer, can induce RET/PTC rearrangements and consequent development of PTC, particularly in the pediatric population (Nikiforov, 2006). No environmental risk factor is known to cause epigenetic alterations in thyroid cancer. Smoking is a well known risk factor in many cancers. Smoking has also been widely shown to be a significant risk factor for the development of thyroid nodule/goiter, particularly in women (Lio et al., 1989; Ericsson and Lindgarde, 1991; Galanti et al., 2005). Thyroid nodule/goiter is, in turn, a strong risk factor for thyroid cancer (Preston-Martin et al., 1993; Franceschi et al., 1999). Although some studies did not show an increased risk associated with smoking for thyroid cancer (Ron et al., 1987; Kreiger and Parkes, 2000; Bandurska-Stankiewicz et al., 2011), others did show an association of smoking with occurrence of thyroid cancer (Sokic et al., 1994). Interestingly, smoking has been found to be associated with aberrant gene methylation in some cancers, such as the *RARβ2* gene methylation in human lung cancer (Tomizawa et al., 2004) and animal lung cancer models (Vuillemenot et al., 2004). The effect of smoking on

gene methylation has not been explored in thyroid cancer. In the present study, we investigated the relationship of smoking with methylation of several major tumor suppressor genes in PTC.

MATERIALS AND METHODS

PATIENTS

With Institutional Review Board approval and, where required, informed patient consent, we included 138 patients (99 female and 39 male, age 15–85 years), who were operated for PTC between 1990 and 2006 and had recorded history of smoking status, in this study. These included 96 patients as never smokers and 42 patients as ever smokers. The latter included current and former smokers. The smoking history of a patient was obtained through a retrospective review of the patient's records. Smoking histories of the ever smokers varied from 2 to 35 pack years of cigarette smoking. A pack-year is defined as equivalent to smoking one pack of cigarettes daily for a period of 1 year.

METHYLATION ANALYSIS

Paraffin-embedded PTC tumors were microdissected and processed and genomic DNA was isolated as described previously (Hu et al., 2006). Bisulfite-treated DNA was subjected to methylation analysis for the promoters of the tumor suppressor genes *TIMP3*, *SLC5A8*, *DAPK*, and *RAR β 2* using real-time quantitative methylation-specific PCR. The primers for these genes and PCR reaction conditions were as described previously (Hu et al., 2006). Any detectable level of methylation for the indicated genes defined as a positive methylation result and zero value on the current detection system was defined as a negative methylation result.

STATISTICAL ANALYSIS

Categorical data were summarized with frequencies and percentages, and continuous data with medians and ranges. Age and gender distribution were analyzed utilizing Mann–Whitney rank sum test and Fisher's exact test, respectively. Smoking status groups were compared using Fisher's exact test for categorical data, and the non-parametric Wilcoxon rank sum test for continuous measures. All reported *P* values are two-sided. A *P*-value of <0.05 was considered to be statistically significant. Analysis was performed using SAS version 9.1.3 software (SAS Institute Inc., Cary, NC, USA).

RESULTS

We analyzed 138 PTC for the methylation status of the tumor suppressor genes *TIMP3*, *SLC5A8*, *DAPK*, and *RAR β 2*. These genes were chosen for analysis because they are important human tumor suppressor genes and their methylation was frequently found in thyroid cancer (Hoque et al., 2005; Hu et al., 2006). Overall, methylation of these genes were detected in 67 (48.5%), 36 (26.1%), 40 (29.0%), and 23 (16.7%) of the 138 cases, respectively. Age and gender were potential confounding variables that could affect gene methylation. As shown in Table 1, except for the age of the *DAPK* methylation-positive patients that was significantly older in the ever-smoker group than the never-smoker group, there was no significant difference in the age of the methylation-positive patients for the remaining three genes between the ever-smoker and never-smoker groups. Similarly, as shown in Table 2, there was

Table 1 | Comparison of mean ages of methylation-positive patients for the indicated genes between the ever-smoker and never-smoker groups.

Genes	Mean age		<i>P</i> value
	Ever smoker	Never smoker	
<i>RARβ2</i>	53.4 \pm 17.6	48.7 \pm 18.2	0.456
<i>DAPK</i>	54.9 \pm 12.4	44.9 \pm 14.9	0.037
<i>TIMP3</i>	54.2 \pm 13.4	47.1 \pm 15.9	0.097
<i>SLC5A8</i>	55.4 \pm 13.7	45.3 \pm 14.9	0.064

Table 2 | Gender distribution of methylation-positive patients for the indicated genes [n (%)] (% = *n*/*N*).

Genes	Female <i>n</i> = 99	Male <i>n</i> = 39	<i>P</i> value
<i>RARβ2</i>	15 (15)	8 (20)	0.45
<i>DAPK</i>	30 (30)	10 (26)	0.68
<i>TIMP3</i>	50 (50)	17 (44)	0.57
<i>SLC5A8</i>	30 (30)	6 (15)	0.09

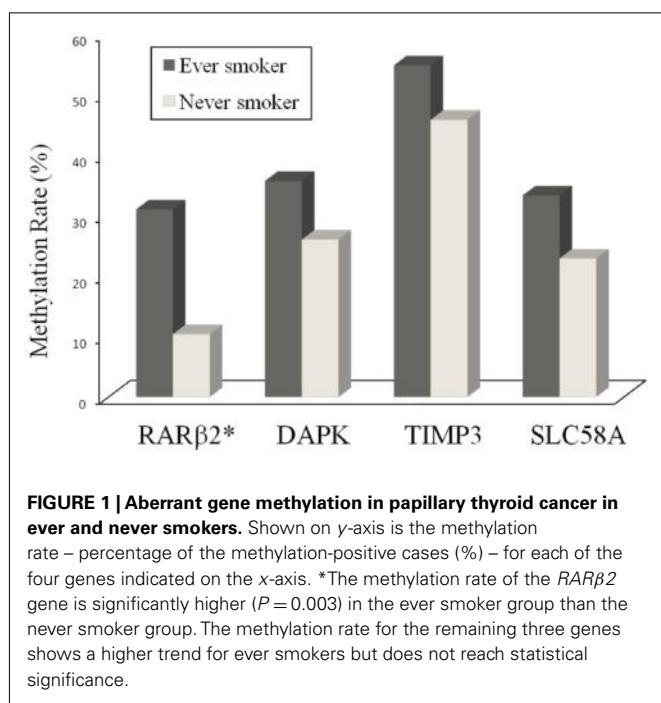
no significant difference in gender distribution of methylation-positive patients for the four genes examined in the present study. Also, smoking did not affect the relationship of gene methylation with tumor size and other tumor characteristics (data not shown).

We next analyzed the relationship of gene methylation in PTC with cigarette smoking history in 138 patients. As summarized in Table 3 and Figure 1, among the four tumor suppressor genes analyzed, methylation of the *RAR β 2* gene was significantly associated with a history of smoking. Specifically, 13/42 (31.0%) ever smokers vs. 10/96 (10.4%) never smokers harbored methylation in the promoter of the *RAR β 2* gene (*P* = 0.003). Although there was no statistically significant difference in the methylation of the *TIMP3*, *SLC5A8*, and *DAPK* genes between the never and ever smokers, there was a tendency of higher prevalences of methylation in these genes in the smokers (Figure 1; Table 3).

The 138 cases of PTC included 72 conventional PTC, 51 follicular variant PTC, and 15 tall-cell PTC. We also analyzed the relationship of smoking with gene methylation in each of the subtype groups of PTC. As shown in Table 3, similar to the results on the overall analysis of all the cases of PTC, *RAR β 2* methylation was significantly associated with smoking [8/17 (47%) of ever smokers vs. 7/55 (13%) of never smokers, *P* = 0.005] in conventional PTC. In the smaller number of cases of follicular variant PTC, marginally significant association of smoking with *RAR β 2* methylation was demonstrated (*P* = 0.06). This relationship was not seen in patients with tall-cell PTC, probably due to the small number of cases. A trend of association of smoking with the *TIMP3*, *SLC5A8*, and *DAPK* methylation was observed in these subtypes of PTC. The average methylation levels for each gene in the ever and never smokers were not significantly different (Table 4). However, this result was likely limited by the inability of the methylation PCR approach used in the present study to accurately provide quantitative measurements.

Table 3 | Relationship of methylation of the *RAR β 2*, *DAPK*, *TIMP3*, and *SLC5A8* genes in papillary thyroid cancer (PTC) with smoking of patients [n (%)].

Tumors	Genes	Ever smoker	Never smoker	P value
All PTC	<i>n</i>	42	96	
	<i>RARβ2</i>	13 (31.0)	10 (10.4)	0.003
	<i>DAPK</i>	15 (35.7)	25 (26.0)	0.25
	<i>TIMP3</i>	23 (54.8)	44 (45.8)	0.33
	<i>SLC5A8</i>	14 (33.3)	22 (22.9)	0.20
Conventional PTC	<i>n</i>	17	55	
	<i>RARβ2</i>	8 (47)	7 (13)	0.005
	<i>DAPK</i>	8 (47)	18 (33)	0.28
	<i>TIMP3</i>	12 (71)	30 (55)	0.24
	<i>SLC5A8</i>	8 (47)	15 (27)	0.13
Follicular variant PTC	<i>n</i>	19	32	
	<i>RARβ2</i>	4 (21)	1 (3)	0.06
	<i>DAPK</i>	3 (16)	2 (6)	0.35
	<i>TIMP3</i>	7 (37)	7 (22)	0.25
	<i>SLC5A8</i>	2 (11)	2 (6)	0.62
Tall-cell PTC	<i>n</i>	6	9	
	<i>RARβ2</i>	1 (17)	2 (22)	1.0
	<i>DAPK</i>	4 (67)	5 (56)	1.0
	<i>TIMP3</i>	4 (67)	7 (78)	1.0
	<i>SLC5A8</i>	4 (67)	5 (56)	1.0

**FIGURE 1 | Aberrant gene methylation in papillary thyroid cancer in ever and never smokers.** Shown on y-axis is the methylation rate – percentage of the methylation-positive cases (%) – for each of the four genes indicated on the x-axis. *The methylation rate of the *RAR β 2* gene is significantly higher ($P = 0.003$) in the ever smoker group than the never smoker group. The methylation rate for the remaining three genes shows a higher trend for ever smokers but does not reach statistical significance.

DISCUSSION

Tobacco smoking has long been known to be a significant risk factor for human cancers. This has prompted numerous studies on its role in molecular derangements in cancers, including genetic and epigenetic alterations, such as aberrant gene methylation. Smoking has also been shown to be associated with the development

Table 4 | Comparison of methylation levels for the indicated genes in papillary thyroid cancer between ever and never smokers.

Genes	Mean methylation levels		P value
	Ever smoker	Never smoker	
<i>RARβ2</i>	0.374 ± 0.73	3.205 ± 8.47	0.067
<i>DAPK</i>	8.565 ± 18.62	3.428 ± 5.82	0.252
<i>TIMP3</i>	5.254 ± 4.59	5.375 ± 7.71	0.222
<i>SLC5A8</i>	31.145 ± 51.26	26.219 ± 39.15	0.783

of thyroid nodules in many studies (Lio et al., 1989; Ericsson and Lindgarde, 1991; Galanti et al., 2005), a strong risk factor for thyroid cancer (Preston-Martin et al., 1993; Franceschi et al., 1999), and occurrence of thyroid cancer in some studies (Sokic et al., 1994). However, little is known about molecular derangements associated with smoking in thyroid cancer. In the present study, we for the first time investigated the relationship between patient smoking and aberrant gene methylation in PTC, the most common type of thyroid cancer. We found a significant association of smoking with methylation of the *RAR β 2* gene in PTC, both in overall PTC and in its major subtypes. This association represented a specific epigenetic event in PTC as the association of smoking with the methylation of other genes (*TIMP3*, *SLC5A8*, and *DAPK*) was not significant. Some of the relationship patterns of smoking with methylation of tumor suppressor genes in PTC observed in the present study were similar to the findings in other cancers. For example, as in the present study which failed to show a significant association of *DAPK* methylation with smoking in PTC, such an association was also not found in non-small lung cancer patients (Liu et al., 2007). Similar to our findings in PTC, previous studies demonstrated a significant association of smoking with *RAR β 2* methylation in lung cancers in humans (Tomizawa et al., 2004) and in animal models (Vuillemenot et al., 2004). Interestingly, even in normal non-cancerous epithelium of upper aerodigestive tract, methylation of the *RAR β 2* gene and other genes was more frequently seen in smokers (Zochbauer-Muller et al., 2003), supporting a direct effect of smoking on gene aberrant methylation. Frequent methylation of the *RAR β 2* gene has also been recently detected in laryngeal squamous cell carcinomas (Paluszczak et al., 2011), a malignancy often associated with smoking.

We previously demonstrated that methylation of the tumor suppressor gene *RAR β 2* was associated with its silencing and, conversely, un-methylation was associated with expression of this gene in thyroid cancer cells (Hu et al., 2006). Re-expression of *RAR β 2* using demethylating agent 5-aza-2'-deoxycytidine caused a significant inhibition of thyroid cancer cell growth (Miasaki et al., 2008). The association of smoking with *RAR β 2* methylation in PTC suggests that this gene may more commonly loose function in PTC of smokers. *RAR β 2* is a major type of retinoic acid (RA) receptors, which are transcription factors that bind with the biologically active metabolites of vitamin A to regulate cell growth and proliferation (Pfahl and Chyttil, 1996). Therapeutic potential of vitamin A and retinoic acid has been demonstrated in various human cancers. RA has been shown to

be able to re-differentiate thyroid cancer cell lines and increase radioiodine uptake in some patients who lost radioiodine avidity (Haugen et al., 2004; Coelho et al., 2005). However, the overall treatment efficacy is poor. One cause for the failure of RA therapy for thyroid cancer is the loss of RA receptor expression in this cancer. *In vitro* studies showed that RA could only inhibit the growth of thyroid cancer cell lines that expressed RAR β and some other RA receptors (Haugen et al., 2004; Elisei et al., 2005). Expression of RA receptors is often decreased or lost in thyroid cancers, particularly in PTC in which expression of the RAR β gene was most frequently decreased or silenced (Tang et al., 2003; Elisei et al., 2005). Our previous demonstration of aberrant RAR β 2 gene methylation in thyroid cancer provided

a molecular explanation (Hoque et al., 2005; Hu et al., 2006). Given these data and the results in the present study, it is clear that aberrant methylation of RA receptor genes is an important molecular event in PTC associated with smoking. How this aberrant methylation of RAR β 2 gene specifically contributes to the tumorigenesis of PTC in smokers warrants further studies to elucidate.

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Poorly differentiated thyroid carcinoma: an incubating entity

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Estimates for 2012 reveal thyroid cancer as the fifth most expected malignancy in American women. Although thyroid cancer is not infrequently diagnosed, it rarely bests its host. We understand a great deal about well-differentiated thyroid cancers, including carcinomas of the thyrocyte and parafollicular c-cells. We have identified a number of mutations and gene rearrangements responsible for familial and sporadic tumors. We have postulated about mechanisms of spread and are able to predict biological behaviors of particular cancer types. However, gray zones remain, and as physicians and scientists, we are never comfortable with these gray zones, as they potentially contain magical epitopes for disease eradication and serve as pesky reminders that medicine is not an exact science. In thyroid cancer, poorly differentiated thyroid carcinomas (PDTC) represent the bridge between more well differentiated malignancies of thyrocytes and the undifferentiated (anaplastic) thyroid carcinomas. Six years ago in Turin, a group of expert surgical pathologists set the stage for refining the diagnosis of PDTC, as introduced by the WHO in 2004, rather than it serving as a diagnosis for those entities which had “high grade features” but did not fit neatly into one of the other defined categories. The conundrum that now exists is the incubation period between the birth of this now well-defined category of thyroid malignancy and the application of the diagnostic criteria, outcomes going forward, and how this diagnosis becomes translated into action by clinicians treating the entity.

WORLD HEALTH ORGANIZATION CLASSIFICATION OF ENDOCRINE TUMORS, 2004

The WHO blue books are the gold standard for diagnostics in surgical pathology. Although the literature churns out an abundance of new data and potential classification schemes, these publications of the WHO are the cornerstones of ana-

tomic pathology-driven diagnostics until they are subsequently updated by designated subspecialist pathologists of the next edition. The most recent classification of endocrine tumors was published in 2004, in which poorly differentiated thyroid carcinomas are introduced as a diagnostic entity (Sobrinho-Simões et al., 2004). PDTC are defined as “follicular neoplasms that show limited evidence of structural follicular cell differentiation and occupy both morphologically and behaviorally an intermediate position between differentiated (follicular and papillary carcinomas) and undifferentiated (anaplastic) carcinomas.” This classification has been intended to combine various definitions of PDTC that have been circulating in the literature and diagnostic surgical pathology reports for many years with many overlapping features but varying degrees of broad interpretation (Sakamoto et al., 1983; Carcangiu et al., 1984; Akslen and LiVolsi, 2000; Sobrinho-Simões et al., 2002; Hiltzik et al., 2006).

TURIN, 2006

About 6 years ago, a group of expert thyroid surgical pathologists gathered in Turin, Italy, to finally hash out what would be considered by consensus to be reproducible criteria for the diagnosis of poorly differentiated thyroid carcinoma (Volante et al., 2007). The analyzed cohort consisted of 83 cases assembled from Europe (Italy), Japan, and the United States with a representative panel consisting of 12 thyroid pathologists. After a lively consensus, the published paper reported a diagnostic scheme, represented by the unshaded areas in **Figure 1** (Volante et al., 2007). As part of the diagnostic algorithm, the tumor would first need to be deemed malignant. The growth patterns were those previously associated with PDTC, including solid, trabecular, or insular (STI) growth. The insular growth pattern is most familiar to pathologists and non-pathologists, with

insular carcinoma often thought of as a high grade carcinoma with extensive necrosis, increased mitoses, broad infiltration of the surrounding thyroid tissue and beyond with a poor prognosis (Carcangiu et al., 1984). Solid and trabecular growth are also quite familiar to surgical pathologists, and these two patterns have diverse context. Most solid-patterned thyroid lesions represent follicular adenomas or benign, unencapsulated adenomatous nodules, but they may also represent solid-type papillary carcinoma, follicular carcinoma, or PDTC. Trabecular growth is largely seen in adenomas, and there is a well-known malignant mimic, the hyalinizing trabecular tumor, which is usually small (<1 cm), with a fingerprint-like appearance that may contain frequent intranuclear pseudoinclusions with low biological potential (Thompson, 2011). However, less frequently, trabecular growth, especially in large, cellular lesions, is ominous appearing, and may raise the possibility of a more biologically aggressive lesion.

The Turin criteria for the diagnosis of PDTC are an easy to follow flow chart (**Figure 1**, unshaded), which arrive at a diagnosis following a series of exclusions. The presence of STI growth is essential, and without this pattern, the tumor is immediately excluded from representing PDTC. If STI growth is present, but the tumor shows largely typical nuclear features of papillary thyroid carcinoma (PTC), the tumor is excluded. If no features of PTC are identified, the tumor is assessed for the presence of any of each of mitoses (≥ 3 mitoses per 10 hpf), convoluted nuclei, or necrosis. Any or all of these features is then sufficient for a diagnosis of PDTC. If not, the lesion is deemed to be a well-differentiated follicular thyroid carcinoma.

These criteria appear simple. However, the first criterion present on the flow chart is that the lesion is a “malignant thyroid tumor of follicular cells.” Of note, encapsulation is not a part of the diagnostic scheme.

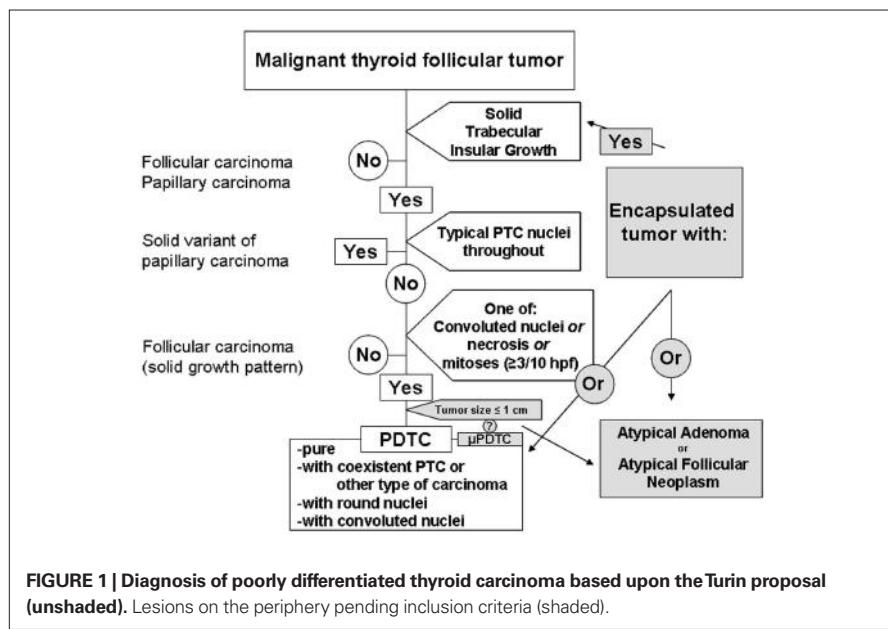


FIGURE 1 | Diagnosis of poorly differentiated thyroid carcinoma based upon the Turin proposal (unshaded). Lesions on the periphery pending inclusion criteria (shaded).

So, even with these clear definitions of PDTC, there exists an additional gray zone, that of the encapsulated follicular tumor with increased mitoses or focal necrosis (Figure 1, shaded). Convoluted nuclei are well-defined by the Turin consensus, but convoluted nuclei alone, without the aid of increased mitoses or necrosis, might be viewed as a subjective challenge to non-thyroid pathology experts. Additionally, how should pathologists interpret solitary lesions that meet the Turin criteria but are subcentimeter?

ENCAPSULATED LESIONS

As mentioned, some encapsulated lesions involve a diagnostic gray zone, as benign lesions, due to size, duration, or external stimuli, may contain features of PDTC. However, depending upon the subjective interpretation of the tumor's malignancy, there is room for broad interpretation. Is the tumor malignant because there is necrosis and mitoses? Or, is the tumor malignant, and therefore the presence of mitoses and/or necrosis appoints that lesion to be a PDTC? We know of encapsulated thyroid carcinomas, specifically, follicular variant of papillary carcinoma, that do not require capsular invasion to qualify as malignant. Biological potential aside, is an encapsulated lesion with convoluted nuclei, necrosis, or increased mitoses a PDTC or an atypical neoplasm of uncertain biological potential

(Figure 1, shaded; Rivera et al., 2010a)? This is unclear and untested, waiting to be fleshed out by retrospective, prospective, and collective data.

STATE OF THE UNION

Incidence of thyroid carcinomas is increasing, with 2012 expected to have thyroid carcinoma as the fifth leading cause of cancer in women (Siegel and Jemal, 2012). Improved classification will require more detailed information for treatment and outcome, with PDTC portending an intermediate prognosis between well-differentiated and undifferentiated thyroid neoplasms (Siironen et al., 2010). Since the Turin proposal, studies have emerged using these new criteria. In a single molecular study, 65 cases selected by Turin criteria were analyzed for mutations in *BRAF*, *N*, *K*, and *HRAS*, as well as translocation of *RET/PTC1, 3*, and *PAX8/PPARγ*. The majority of mutations (15/65) were found in *RAS* (largely *NRAS*), with a single mutation found in *BRAF* (V600E). The single *BRAF* mutation was found in a PDTC associated with tall cell PTC (Volante et al., 2009). No other mutations were identified. This same paper looked at p53 by immunohistochemistry, with expression in ~30% of tumors, but accounting for less than 20% of tumor staining, making mutated *TP53* unlikely to be a major contributor. *TP53* is well-known to be mutated in undifferentiated (anaplastic) carcinomas (Lavra et al.,

2009) and though reported or discussed in other cohorts of PDTC (Malaguarnera et al., 2007; Nambiar et al., 2011), it has not been well-studied in a significant post-Turin cohort. Some estimates have placed mutations here at about 20–30% (Nikiforov and Nikiforova, 2011), but most of the large *TP53* mutational studies were performed in the early to mid-1990s (Donghi et al., 1993; Fagin et al., 1993; Dobashi et al., 1994). In an additional large study with collaborating authors from the aforementioned group, this cohort validated the Turin criteria in a retrospective fashion using cases from the United States and Italy, showing the addition of one molecular/immunohistochemical marker, *IMP3*, to be an indicator of poor prognosis in PDTC (Asioli et al., 2010). The study also reiterated *NRAS* to be most commonly mutated in PDTC, including the prior study (about 20% of cases).

SPECIFICITY OF DIAGNOSIS IN PDTC

Ultimately, the recent reclassification of PDTC is intended to improve patient care, with more uniform, accurate reporting. Importantly, the criteria exist to link objective findings with clinical outcomes, biological potential, and hopefully, following additional study, molecular mechanisms with targeted treatment modalities and improved outcomes. For instance, oncocytic neoplasms were excluded in the Turin proposal. However, in a recent validation by the Mayo group in collaboration with the Turin group, about 1/3 of tumors had oncocytic features (Asioli et al., 2010). Also, a very recent study reviewing a large cohort of aggressive thyroid carcinomas (129 with known recurrence or death) identified 18 oncocytic PDTC (Dettmer et al., 2012). However, this group does not distinguish the 18 oncocytic PDTC from other oncocytic (Hürthle cell) carcinomas present among the 129 cases, and the majority (16/18) of these cases were included due to necrosis, in addition to STI growth pattern. What constitutes convoluted nuclei in this group (18/18), with oncocytic nuclei generally thought of as round with prominent, centrally located nucleoli, is unclear.

MISSING LINK

Well-differentiated thyroid carcinomas of follicular origin, including PTCs and follicular thyroid carcinomas, have several known mutations or translocation events.

PTCs have mutations in *BRAF*, occasional *RAS* subtypes, and may have translocations of *RET/PTC* 1,2 (Nikiforov and Nikiforova, 2011). Follicular carcinomas can have aberrations of *RAS* or *PAX8/PPAR γ* (Nikiforov and Nikiforova, 2011). Additional exceptions exist, but these are certainly most common for those who work with patients with thyroid cancer. Undifferentiated (anaplastic) thyroid carcinomas (ATC), in addition to possible prior *BRAF* or *RAS* mutations, may harbor additional mutations in *TP53* or beta-catenin (*CTNNB1*; Nikiforov and Nikiforova, 2011). This mechanism of transformation is thought to be a multi-hit hypothesis, as ATC with associated more well-differentiated components have been shown to have mutations in *BRAF* in the well-differentiated component, with the additional *CTNNB1* or *TP53* mutation seen only in the undifferentiated areas (Nikiforov, 2004; Quiros et al., 2005).

However, this is not how we generally encounter these cancers. Most, seen microscopically, do not have a nice transition for us to identify tumor origin. Additionally, we may discover late recurrences of well-differentiated tumors as something more sinister, often after some treatment modality other than surgical excision.

WELL-DIFFERENTIATED CARCINOMAS WITH HIGH GRADE FEATURES

We would like to briefly discuss the concept of a well-differentiated carcinoma with high grade features. Although papillary-type PDTC can show concurrent cancer types, many cancers exist as majority PTCs with foci of high grade changes, such as necrosis, focal loss of papillary-type nuclei, and foci with overall aberrant morphology (Rivera et al., 2010a,b). Widely invasive follicular or Hürthle cell carcinoma may have associated foci showing necrosis or increased mitoses, and by their nature, they are often associated with the STI growth pattern. Likely, this is why so many of the subsequently studied tumors post-Turin have *RAS* mutations (Ricarte-Filho et al., 2009; Volante et al., 2009; Asioli et al., 2010). This tumor is not entirely poorly differentiated, as you can see a more well-differentiated component to it, but drawing a definitive diagnostic line between FTC and PDTC remains challenging. The truth is that we call these tumors many things and often diagnose them descriptively, presenting

challenges for our clinical colleagues, who would like to rely more and more often on molecular data for individualized care and potential targeted therapies, some of which have been approached with animal models (Nucera et al., 2010; Chakravarty et al., 2011), whereas some authors propose we scrap our current classification system entirely (Kakudo et al., 2012). Is this descriptive diagnosis preventing an appropriate accounting of disease incidence, or are we displaying the limits of our current knowledge base while additional molecular and proteomic data are accrued? We would argue for the latter.

VALIDATION

As the Turin criteria were released 5 years ago (Volante et al., 2007), there have been a few published validation studies (Volante et al., 2009; Asioli et al., 2010), including a recent study of oncocytic PDTC (Dettmer et al., 2012), controversial in that oncocytic tumors were excluded from the Turin study set. The majority of the discussion of the new criteria for PDTC is from a respectable cadre of experts (Bongiovanni et al., 2009; Garcia-Rostan and Sobrinho-Simões, 2010; Volante and Papotti, 2010; Dettmer et al., 2011; Nambiar et al., 2011; Tallini, 2011).

CONCLUSION

The past decade has seen a shift in diagnosis of follicular-patterned thyroid lesions, including high grade lesions. The publication in 2004 of the WHO blue book on endocrine tumors introduced poorly differentiated thyroid carcinoma as a distinct diagnostic entity. This was further well-delineated in Turin in 2006, providing tangible criteria for the everyday practicing pathologist to diagnose this elusive, boutique entity. As we continue to gather data, the current classification scheme will either be solidified or discarded, with continuing attention paid to molecular changes and subsets of lesions. Clinicians treating patients with PDTC will need to become familiar with the diagnostic criteria for this diagnosis, constructing appropriate treatment modalities and follow up for these patients. With additional experience, the diagnostic model will be tested and ultimately found to be clinically relevant; and if not, it will be discarded for something deemed to be more rational.

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RET/PTC translocations and clinico-pathological features in human papillary thyroid carcinoma

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Thyroid carcinoma is the most frequent endocrine cancer accounting for 5–10% of thyroid nodules. Papillary histotype (PTC) is the most prevalent form accounting for 80% of all thyroid carcinoma. Although much is known about its epidemiology, pathogenesis, clinical, and biological behavior, the only documented risk factor for PTC is the ionizing radiation exposure. Rearrangements of the Rearranged during Transfection (RET) proto-oncogene are found in PTC and have been shown to play a pathogenic role. The first *RET* rearrangement, named *RET/PTC*, was discovered in 1987. This rearrangement constitutively activates the transcription of the *RET* tyrosine-kinase domain in follicular cell, thus triggering the signaling along the MAPK pathway and an uncontrolled proliferation. Up to now, 13 different types of *RET/PTC* rearrangements have been reported but the two most common are *RET/PTC1* and *RET/PTC3*. Ionizing radiations are responsible for the generation of *RET/PTC* rearrangements, as supported by *in vitro* studies and by the evidence that *RET/PTC*, and particularly *RET/PTC3*, are highly prevalent in radiation induced PTC. However, many thyroid tumors without any history of radiation exposure harbor similar *RET* rearrangements. The overall prevalence of *RET/PTC* rearrangements varies from 20 to 70% of PTCs and they are more frequent in childhood than in adulthood thyroid cancer. Controversial data have been reported on the relationship between *RET/PTC* rearrangements and the PTC prognosis. *RET/PTC3* is usually associated with a more aggressive phenotype and in particular with a greater tumor size, the solid variant, and a more advanced stage at diagnosis which are all poor prognostic factors. In contrast, *RET/PTC1* rearrangement does not correlate with any clinical-pathological characteristics of PTC. Moreover, the *RET* protein and mRNA expression level did not show any correlation with the outcome of patients with PTC and no correlation between *RET/PTC* rearrangements and the expression level of the thyroid differentiation genes was observed. Recently, a diagnostic role of *RET/PTC* rearrangements has been proposed. It can be searched for in the mRNA extracted from cytological sample especially in case with indeterminate cytology. However, both the fact that it can be present in a not negligible percentage of benign cases and the technical challenge in extracting mRNA from cytological material makes this procedure not applicable at routine level, at least for the moment.

Keywords: *RET*, *RET/PTC*, papillary thyroid cancer, oncogene

INTRODUCTION

Thyroid nodules are a very common clinical finding; the prevalence of palpable nodules ranges from 4 to 7% in general population (Mazzaferri, 1992; Gharib, 2004). Although only less than 5% of palpable nodules are malignant lesions, thyroid cancers are the most frequent endocrine malignancy accounting for about 5–10% of thyroid nodules (Braverman and Utiger, 2005). Epidemiological studies in USA and in Europe demonstrated a relevant increased incidence of thyroid cancer and, in particular, thyroid cancer rate of incidence has been the highest among all human tumors during the last decades (Farahati et al., 2004; Davies and Welch, 2006; Kent et al., 2007; Enewold et al., 2009).

About 95% of malignant lesions are derived from thyroid follicular cells and are distinguished into well differentiated,

either papillary (PTC) or follicular (FTC) histotype, and anaplastic thyroid carcinoma (ATC; Cardis et al., 2006). As shown in Figure 1, PTC is the most prevalent form accounting for about 80% of cases, while FTC represent only 15%. PTC and, to a lesser extent, FTC have a good prognosis if adequately treated (Elisei et al., 2010). Very rare (5%), but also very aggressive and almost invariably lethal, is ATC. Another relatively small percentage of thyroid carcinomas (7.5–10%; Schlumberger and Pacini, 1999) are derived from parafollicular C cells and are called medullary thyroid carcinoma (MTC; De Lellis et al., 2003; Nikiforov, 2009).

In the last 25 years, many studies have been conducted to identify the genetic alterations related to the pathogenesis of these tumors. Several oncogenes have been analyzed for mutations and

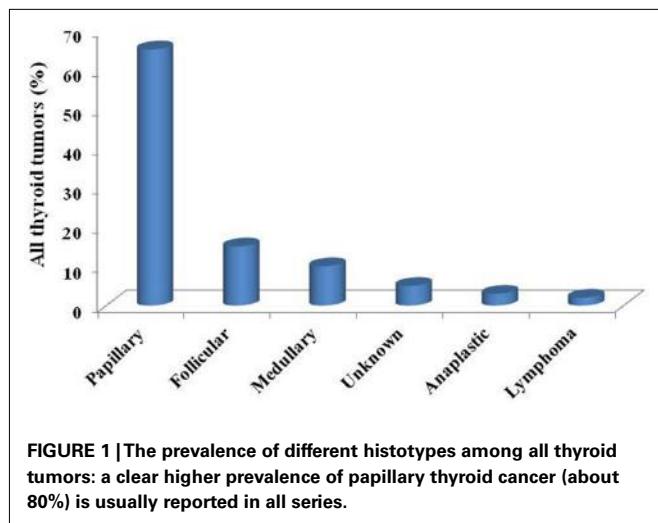


Table 1 |Different prevalences of different oncogenes reported to be involved in thyroid carcinogenesis.

Oncogene	Mean prevalence (%)*
BRAF	48
RET/PTC	20
APC	15
SMAD4	14
CTNNB1	12
IDH1	10
NTRK1	10
CDKN2A	9
EGFR	8
NRAS	4
HRAS	2
KRAS	2
PIK3CA	2

*Data derived from: <http://www.sanger.ac.uk/genetics/CGP/cosmic/>

some of them have been found to be strictly correlated with a specific thyroid carcinoma histotype (Table 1).

In this review we will focus on PTC and in particular on the relationship between the presence of *RET/PTC* rearrangements, that have been defined to be specific for PTC development and the clinical and pathological features of PTC. The putative role of *RET/PTC* rearrangements in the diagnosis and in the prediction of prognosis will be discussed.

PAPILLARY THYROID CANCER

The annual incidence of thyroid cancer per 10^5 individuals ranges from 1.2 to 2.6 in men and from 2.0 to 3.8 in women and tumor mortality is very low (Franceschi et al., 1993). Thyroid cancer is two to four times more frequent in females than in males. It is rare in children below 16 years of age while in adults the incidence increases with age and the median age at diagnosis is between 45 and 50 years. Differences in the incidence of thyroid cancer according to ethnic origin have been also reported. These differences may

be due to environmental factors and dietary habits (Spitz et al., 1988).

Although the etiology of PTC is not well understood, there are some risk factors which are known to put an individual at higher risk of developing the disease. Radiation exposure significantly increases the risk for thyroid malignancies, particularly PTC. This finding was observed in children exposed to radiation after the nuclear bombings in Hiroshima and Nagasaki during the Second World War (Nagataki et al., 1994). Additional evidence was gathered after atomic bombs were tested in the Marshall Islands (Cronkite et al., 1995), after the accident at the Chernobyl nuclear power plant (Williams, 2002; Nikiforov, 2006), and in patients who received low-dose radiation therapy for benign disorders of the head, neck, and thorax (Duffy and Fitzgerald, 1950; Winship and Rosvoll, 1970).

Among other risk factors female gender play an important role in fact it has been shown (Ron et al., 1987) that the sex ratio approaches 1 before puberty and after menopause while during the fertile woman age it is between two and four times higher than in the correspondent men age period. A heavier body weight have been shown also to increase the risk of thyroid cancer (Kitahara et al., 2011).

An important role is also played by the iodine exposure. Areas with low iodine intake usually show higher prevalence of FTC than area with sufficient iodine intake where, conversely, the PTC histotype is more represented (Belfiore et al., 1987). Furthermore the high prevalence of thyroid cancer in regions where the iodine intake is high suggests that iodine intake may play role in thyroid tumorigenesis (Vejbjerg et al., 2007; Guan et al., 2009) as well as other factors not related to iodine like volcanic activity (Pellegriti et al., 2009).

RET/PTC REARRANGEMENTS

The *RET* proto-oncogene is located on chromosome 10q11.2 and encodes for a transmembrane tyrosine-kinase receptor involved in the control of cell differentiation and proliferation. Four different ligands have been reported up to now: glial derived neurotrophic (GDN) factors, Neurturin (NRTN), Artimin (ARTN), and Persepin (PSPN), respectively (Arighi et al., 2005). All these ligands induce *RET* activation through the binding to specific coreceptors. The *RET* gene is expressed in tissues deriving from the neural crest including thyroid C cells and adrenal medulla but it is not expressed in normal thyroid follicular cells (Santoro et al., 1990).

In 1987 the first finding of a new oncogene activated in human PTC was reported (Fusco et al., 1987). Interestingly, the tumoral tissue where the oncogene was firstly described was derived from an irradiated PTC. After 3 years, this oncogene was molecularly cloned: it was a chimeric gene generated by the fusion of the *RET* tyrosine-kinase domain (Wirtschafter et al., 1997) with the 5' terminal region of a new gene denominated CCD6 (formerly called H4; Figure 2; Grieco et al., 1990). This oncogene was named *RET/PTC*.

After the first identification, several types of *RET/PTC* rearrangements have been described (Greco et al., 2009; Table 2). At the present 13 different types of *RET/PTC* rearrangements have been reported and all of them are the result of the fusion of the

RET tyrosine-kinase (TK) domain with different genes, which are characterized by the presence of nucleotide sequences coding for proteins with an extremely high probability of forming coiled-coil domains, thus allowing constitutive dimerization of the RET-TK domain. This constitutive dimerization determines an uncontrolled proliferation of the follicular cells harboring the RET/PTC rearrangement and the development of malignancy. The presence of RET/PTC rearrangement in microcarcinoma strongly support the hypothesis of a driving role of this oncogene in the tumoral transformation (Viglietto et al., 1995).

So far, RET/PTC rearrangements have been identified almost exclusively in thyroid lesions, and in particular in PTC (Santoro et al., 1993; Tallini and Asa, 2001; Nikiforov, 2002). To our knowledge, the only other human tumor harboring RET/PTC rearrangements is primary peritoneal carcinoma (Flavin et al., 2009). However these rearrangements are supposed to be

“passenger” mutations reflecting RET instability in tumor subclones more than true pathogenetic events. RET/PTC rearrangements more frequently found in PTC are RET/PTC1, given by the fusion with the CCDC6 (formerly H4) gene and RET/PTC3, given by the fusion with the NCOA4 (formerly ELE1) gene (Santoro et al., 1994).

The reported RET/PTC prevalence in thyroid tumors varies greatly in different series (Zou et al., 1994; Lam et al., 1998; Mayr et al., 1998; Chua et al., 2000; Cinti et al., 2000; Fenton et al., 2000; Sheils et al., 2000; Puxeddu et al., 2003; Rhoden et al., 2004, 2006; **Table 3**). This difference can be attributed to ethical and geographic variations as well as to the different sensitivities of detection methods (Zhu et al., 2006). It has been carefully demonstrated that the method used has an important effect on the efficacy of RET/PTC rearrangement determination and thus on the reported prevalence (Marotta et al., 2011). Tumor heterogeneity is another factor that can affect the evaluation of RET/PTC prevalence. The distribution of RET/PTC rearrangement within the same tumor may be heterogeneous, varying from the involvement of most neoplastic cells (i.e., clonal RET/PTC) to the presence of

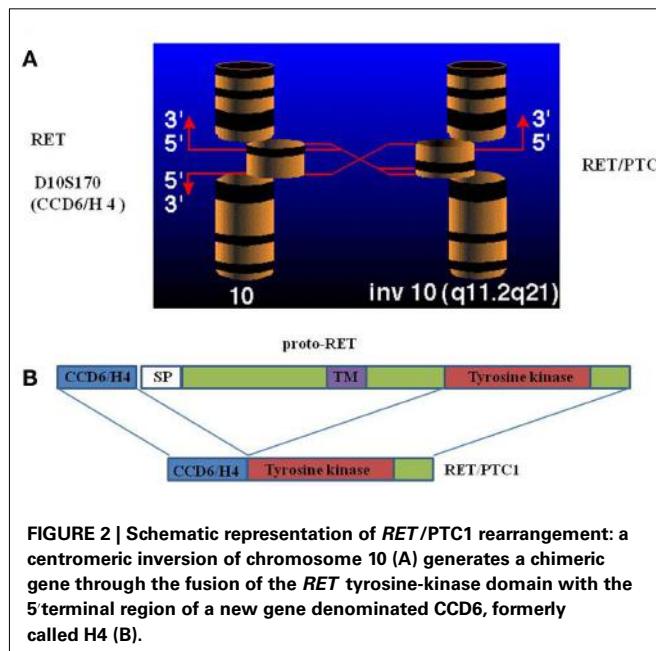


Table 2 | Different types of RET/PTC rearrangements in thyroid tumors.

Oncogene	Donor gene	Chromosomal location
RET/PTC1	CCDC6 (formerly H4)	10q21
RET/PTC2	PRKAR1A	17q23
RET/PTC3	NCO4 (formerly Ele 1)	10q11.2
RET/PTC4	NCO4 (formerly Ele 1)	10q11.2
RET/PTC5	Golgas	14q
RET/PTC6	TRIM24	7q32-34
RET/PTC7	TRIM33	1p13
RET/PTC8	KTN1	14q22.1
RET/PTC9	RFG9	18q21-22
ELKS-RET	ELKS	12p13.3
PCM1-RET	PCM1	8p21-22
RFP-RET	TRIM27	6p21
HOOK3-RET	HOOK3	8p11.21

Reference	Post-Chernobyl n (%)	Spontaneous n (%)
Guerra et al. (2011)**	nd	36%
Hieber et al. (2011)	16/22 (72)	nd
Hamatani et al. (2008)	11/50 (22)*	nd
Rhoden et al. (2006)	nd	25/34 (73)
Zhu et al. (2006)	nd	26/75 (34)
Unger et al. (2006)	10/13 (77) [°] 9/32 (28) ⁺	nd
Di Cristofaro et al. (2005)	11/17 (65)	9/21 (43)
Rhoden et al. (2004)	nd	18/25 (72)
Puxeddu et al. (2003)	nd	13/48 (27)
Elisei et al. (2001)	19/25 (76)	11/47 (23)
Cinti et al. (2000)	nd	13/69 (19)
Sheils et al. (2000)	nd	12/50 (24)
Fenton et al. (2000)	nd	15/33 (45)
Chua et al. (2000)	nd	44/62 (71)
Thomas et al. (1999)	37/67 (55)	nd
Smida et al. (1999)	25/51 (49)	nd
Mayr et al. (1998)	nd	8/99 (8)
Tallini et al. (1998)	nd	81/201 (40)
Lam et al. (1998)	nd	17/40 (43)
Sugg et al. (1999)	nd	51/86 (59)
Nikiforov et al. (1997)	33/38 (87)	12/17 (70)
Klugbauer et al. (1995)	9/15 (60)	nd
Fugazzola et al. (1995)	4/6 (66)	nd
Zou et al. (1994)	nd	1/40 (2.5)
Ishizaka et al. (1991)	nd	1/11 (9)

*Atomic bomb survivors.

⁺Long latency period.

[°]Short latency period.

**Data obtained on cytological samples.

a small fraction of tumor cells (i.e., non-clonal *RET/PTC*) in the sample (Unger et al., 2004; Zhu et al., 2006).

Clonal *RET/PTC* rearrangements occur in about 20% of PTC and are specific for this tumor (Nikiforov, 2002, 2011; Zhu et al., 2006). Non-clonal *RET/PTC* rearrangements have been found not only in PTC but also in 10–45% of thyroid adenomas and other non-neoplastic thyroid lesions (Ishizaka et al., 1991; Wirtschafter et al., 1997; Sheils et al., 2000; Elisei et al., 2001; Chiappetta et al., 2002; Sapiro et al., 2011) and Hashimoto's thyroiditis (Rhoden et al., 2006).

As shown in **Table 3**, *RET/PTC* rearrangements are more frequently found in thyroid cancers following radiation exposure (50–80%; Fugazzola et al., 1995; Nikiforov et al., 1997; Thomas et al., 1999; Di Cristofaro et al., 2005; Unger et al., 2006; Hamatani et al., 2008; Hieber et al., 2011). In particular *RET/PTC3* has been found to be more frequent than *RET/PTC1* in post-Chernobyl radiation exposed thyroid cancer especially in those with a short latency period (Williams, 2008). *RET/PTC* rearrangements have been also found to be more prevalent in children than in adults both in irradiated and non-irradiated PTC (Nikiforov et al., 1997; Elisei et al., 2001).

Over the time, the proportion of post-Chernobyl thyroid tumors with a *RET/PTC* rearrangement has declined, and in the *RET/PTC*-positive tumors the percentage of those with *RET/PTC1* has increased while the proportion of those with *RET/PTC3* has in parallel decreased (Unger et al., 2006). The hypothesis to explain this variation is that with the increase of latency period the effect of radiation is becoming less significant and most recently developed PTC around Chernobyl are more similar to non-irradiated cases. This hypothesis is at least in part supported by the evidence that also the histological features are changing according to the latency period (Williams, 2008).

The high prevalence of *RET/PTC* rearrangements found in post-Chernobyl thyroid tumors and in atomic bomb survivors exposed to high-dose radiation (Hamatani et al., 2008) strongly supports a direct role of radiation exposure in *RET/PTC* generation (Fugazzola et al., 1995; Klugbauer et al., 1995; Bounacer et al., 1997; Nikiforov et al., 1997). *RET/PTC* rearrangements were also found after high-dose radiation to human undifferentiated thyroid carcinoma cells (Ito et al., 1993) and to fetal human thyroid tissue transplanted into SCID mice (Mizuno et al., 2000). The generation of *RET/PTC* rearrangements has been also demonstrated in normal thyroid cultured cells after *in vitro* exposure to radiation (Gandhi et al., 2010a,b). Furthermore, the strong relationship between ionizing radiation exposure and *RET/PTC* rearrangements have been shown by a COMET assay that demonstrated that *RET* oncogene is very susceptible to ionizing radiation (Volpatto et al., 2008). Moreover, the spatial contiguity of *RET* and *H4* genes within the chromatin may provide a structural basis for generation of *RET/PTC1* rearrangement by allowing a single radiation track to produce a double-strand break in each gene (Nikiforova et al., 2000; Gandhi et al., 2006, 2010a).

Over the last few decades, significant progress has been achieved in the understanding of the biological mechanisms of radiation carcinogenesis. It has been shown that damage to cellular DNA is responsible for mutagenesis and carcinogenesis and that double-strand breaks is the most important event for the direct

generation of gene translocations and rearrangements. In particular there are several *in vitro* evidences demonstrating that thyroid cells exposed to X-Ray develop *RET/PTC* rearrangements (Ito et al., 1993; Goodhead, 1994; Ward, 1995; Mizuno et al., 2000; Caudill et al., 2005). Other than ionizing radiations, other putative carcinogens, like caffeine, ethanol, hypoxia, and others, are able to induce DNA double-strand breaks and generate *RET/PTC* rearrangements (Gandhi et al., 2010a,b). Recently, a direct relationship between ionizing radiation exposure, intracellular hydrogen peroxidase (H_2O_2) generation, double-strand induction, and *RET/PTC1* rearrangements development was also demonstrated (Ameziane-El-Hassani et al., 2010). After this observation, it can be hypothesize that other agents able to initiate biological production of superoxidase anions and H_2O_2 (Narayanan et al., 1997) can induce *RET/PTC* rearrangements.

RET/PTC REARRANGEMENTS AND CLINICO-PATHOLOGICAL FEATURES OF PTC

A correlation with a more aggressive phenotype and a more advanced stage has been reported for *RET/PTC* rearrangements, especially *RET/PTC3* (Nikiforov et al., 1997; Powell et al., 1998). In particular, in post-Chernobyl childhood thyroid cancer the *RET/PTC3* rearrangement was more frequently associated with the solid variant of PTC which is considered a more aggressive variant and the most prevalent among these irradiated tumors. As matter of fact, with the increase of the latency period both the solid variant and the *RET/PTC* rearrangements prevalence have declined suggesting a strong relationship between radiation exposure, solid variant, *RET/PTC3* rearrangements, and a more rapid development of the tumor as indicated by the short latency period (Williams, 2008).

In a previous study on sporadic PTC we also demonstrated a positive correlation between the presence of *RET/PTC3* rearrangement, but not of *RET/PTC1*, with a bigger size of the tumor and a more advanced stage at diagnosis. However, in the same study the levels of expression of thyroid differentiation genes (i.e., thyrotropin stimulating hormone receptor, thyroglobulin, sodium-iodide symporter etc.) was shown to be not significantly different in PTC with or without *RET/PTC* rearrangements, thus suggesting that these genetic alterations should not play a major role in the de-differentiation process (Romei et al., 2008).

Controversial data have been also reported about the correlation of the presence of *RET/PTC* rearrangements and clinical and epidemiological features of patients with PTC. Although there is a general agreement that *RET/PTC* rearrangements, particularly *RET/PTC1*, are more frequent in young patients, also in non-irradiated cases (Elisei et al., 2001) there are other studies that did not show any correlation between *RET/PTC* rearrangements and age, sex, tumor size, staging, number of neoplastic foci, and histological subtype (Puxeddu et al., 2004).

So far no consensus concerning the clinical prognostic value of the presence of a *RET/PTC* rearrangement, either *RET/PTC3* or *RET/PTC1*, has been reached. Some evidences exists suggesting that PTC with *RET/PTC1* rearrangement are associated with a more favorable behavior (Saad et al., 2004) while those harboring *RET/PTC3* rearrangement may be more prone to de-differentiation and a more aggressive behavior (Sugg et al., 1999;

Mochizuki et al., 2010). Initial reports on this specific issue claimed a role in metastatic spread (Jhiang and Mazzaferri, 1994; Sugg et al., 1999) and more recently, it has been suggested that *RET/PTC*-positive cases show higher rates of local extension and lymph node involvement than *RET/PTC* negative cases (Adeniran et al., 2006). At variance, several other studies were unable to find any relationship between *RET/PTC* rearrangements and classical prognostic factors (Tallini et al., 1998; Musholt et al., 2000; Basolo et al., 2001; Romei et al., 2008).

In our experience, *RET/PTC* rearrangements do not seem to be correlated with any clinical and pathological feature of aggressiveness either by studying the expression of the *RET/PTC* protein by immunohistochemistry (Basolo et al., 2001) or by studying the mRNA expression of the *RET/PTC* rearrangements by real time RT-PCR (Romei et al., 2008).

DIAGNOSTIC ROLE OF *RET/PTC* REARRANGEMENTS

Fine needle aspiration cytology (FNAC) is the first choice method to distinguish between benign and malignant thyroid nodules (Pacini et al., 2006; Baloch and LiVolsi, 2008; Cooper et al., 2009). However, about 30% of FNAC yields uncertain results because of inadequate sampling (i.e., only few cells or great blood contamination) or because cytological features do not clearly indicate the nature of the lesion (i.e., follicular neoplasia; Gharib et al., 1984; Mazzaferri, 1993). Whenever a follicular neoplasia is diagnosed, surgical treatment, and histological examination are required to differentiate the malignant from the benign nature of the nodule. Since only 20–30% of these nodules turn out to be malignant (Rago et al., 2007), about 80% of patients with a follicular neoplasia undergo an unnecessary thyroidectomy.

The rapidly expanding knowledge of molecular genetics of thyroid cancer has started to translate into clinical practice, offering significant improvement in accuracy of the preoperative diagnosis of thyroid cancer. Several studies have been already published on the possibility to improve the diagnostic power of FNAC by looking for thyroid cancer specific oncogenic alterations (Nikiforova and Nikiforov, 2009). Most studies have explored the diagnostic role of *BRAF* mutation (Jin et al., 2006; Xing et al., 2009; Adeniran et al., 2011; Marchetti et al., 2012). In a large prospective study recently published, the detection of *BRAF* mutations in FNAC showed a very high positive predictive value (PPV). However, the biggest diagnostic impact can be achieved only by testing

FNA samples for a panel of mutations (i.e., *BRAF*, *RET/PTC*, *RAS*, *TRK*) rather than for a single mutation (Cantara et al., 2010; Guerra et al., 2011). Indeed, *RET/PTC* detection can improve the preoperative diagnosis of thyroid nodules, particularly in samples that are indeterminate by cytology or have an insufficient amount of cells for cytologic evaluation (Cheung et al., 2001; Salvatore et al., 2004; Pizzolanti et al., 2007). Nevertheless the PPV of the detection of *RET/PTC* rearrangements is not as high as the detection of *BRAF* mutations because a low, but not negligible, percentage of nodules positive for *RET/PTC* rearrangements on FNA which turned out to be benign at histology (Cantara et al., 2010; Guerra et al., 2011). This is not unexpected since, have previously said, there are several reports showing that 5% of benign thyroid nodules and several cases of Hashimoto's thyroiditis are positive for *RET/PTC* rearrangements. Last but not least, the search for *RET/PTC* rearrangements requires the mRNA extraction form the cytological smears that is a much more challenging techniques than the extraction of DNA that is required for the search of *BRAF*. As matter of fact, at the present the search for *RET/PTC* rearrangements in cytologically indeterminate thyroid nodule is still not considered a diagnostic routine tool.

CONCLUSION

In the last years important achievements have been reached in the understanding of the molecular basis of PTC. Although different oncogenes have been found to be altered in PTC, *BRAF* mutations, and *RET/PTC* rearrangements are the most frequently involved. A lot has been discovered between the relationship of *RET/PTC* rearrangements and PTC clinical, pathological, and epidemiological features. Although present also in non-irradiated cases, *RET/PTC* rearrangements are related to radiation exposure and are more frequent in patients with radio induced PTC. Among all *RET/PTC* rearrangements, *RET/PTC1*, and *RET/PTC3* are in general the most frequent. *RET/PTC3* is much more prevalent in irradiated PTC especially in those with solid variants. Both of them are more prevalent in children than in adults. Despite all these observations there are not yet clear data showing a definitive prognostic role of neither *RET/PTC1* or *RET/PTC3*. The presence of a *RET/PTC* rearrangement in the RNAs extracted from cytological material aspirated from a thyroid nodule is strongly predictive of malignancy but some cases of benign nodules can also result to be positive.

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Intracellular signal transduction and modification of the tumor microenvironment induced by RET/PTCs in papillary thyroid carcinoma

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RET gene rearrangements (RET/PTCs) represent together with BRAF point mutations the two major groups of mutations involved in papillary thyroid carcinoma (PTC) initiation and progression. In this review, we will examine the mechanisms involved in RET/PTC-induced thyroid cell transformation. In detail, we will summarize the data on the molecular mechanisms involved in RET/PTC formation and in its function as a dominant oncogene, on the activated signal transduction pathways and on the induced gene expression modifications. Moreover, we will report on the effects of RET/PTCs on the tumor microenvironment. Finally, a short review of the literature on RET/PTC prognostic significance will be presented.

Keywords: thyroid carcinoma, papillary thyroid carcinoma, RET/PTC

INTRODUCTION

Thyroid cancer is the most common malignant tumor of the endocrine system and accounts for approximately 3% of all newly diagnosed cancer cases in the United States¹. The most frequent type of thyroid cancer is papillary thyroid carcinoma (PTC), which constitutes ~80% of all cases. PTC frequently has genetic alterations leading to the activation of the mitogen-activated protein kinase (MAPK) signaling pathway. Those include RET/PTC and *NTRK1* rearrangements and *BRAF* and *RAS* point mutations (Figure 1). Mutations involving one of these genes are found in about 70% of papillary carcinomas and they rarely overlap in the same tumor (Kimura et al., 2003; Puxeddu et al., 2004; Carta et al., 2006; Kondo et al., 2006).

In this review, we will focus on RET/PTC rearrangements summarizing the available data on the molecular mechanisms involved in their formation and in their function as dominant oncogenes, on the activated signal transduction pathways, and on induced gene expression modifications. Moreover, we will report on their effects on tumor microenvironment.

GENERAL FEATURES OF RET/PTCs

The *RET* (REarranged during Transfection) proto-oncogene is located in the pericentromeric region of chromosome 10q11.2 and spans 21 exons (Takahashi et al., 1985). *RET* encodes for a cell membrane receptor tyrosine kinase (TK), which is expressed in neuroendocrine cells (including thyroid C cells and adrenal

medullary cells), neural cells, urogenital tract cells, and testis germ cells (Wells and Santoro, 2009). RET protein displays an extracellular portion which contains four cadherin-like repeats, a calcium-binding site and a cysteine-rich region (Anders et al., 2001), a transmembrane portion and an intracellular portion, which contains the juxtamembrane domain, the TK domain, split in two subdomains by an insert of 14 amino acids, and a C-terminal tail (Knowles et al., 2006). Glial cell line-derived neurotrophic factor, neurturin, artemin, and persephin have been shown to be RET ligands (Durbec et al., 1996; Sariola and Saarma, 2003). They bind RET in conjunction with one of four glycosyl-phosphatidylinositol-anchored co-receptors, designated GDNF-family α receptors (GFR α) 1, 2, 3, and 4 (Puxeddu and Fagin, 2001).

Structurally, RET-TK adopts the typical protein kinase bilobate structure consisting of a small N-lobe and a large C-lobe connected by a short linker (Knowles et al., 2006). However, at variance with most kinases which are monomeric and auto-inhibited *in cis* by the C-lobe activation loop (A-loop) that maintains the kinase pocket closed in unstimulated conditions, RET-TK is already a dimer in the resting state. It adopts a *trans*-inhibited head-to-tail inactive dimer conformation in which the substrate-binding site of each monomer is occluded by the controlateral one (Knowles et al., 2006). While in other kinases ligand binding causes dimerization, rearrangement of the A-loop, and kinase activation, interaction of RET with the GDNF/GFR α complex probably causes a conformational change that relieves the *trans*-inhibition and favors the formation of active dimers (Knowles et al., 2006).

¹http://seer.cancer.gov/csr/1975_2008/

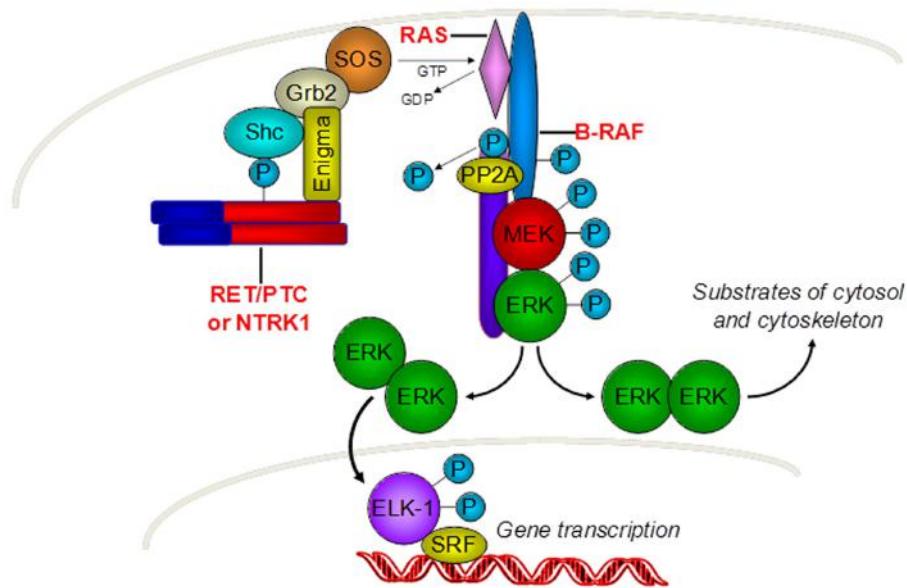


FIGURE 1 | Gene mutations involved in PTC carcinogenesis. PTC is driven by genetic alterations leading to the activation of the mitogen-activated

protein kinase (MAPK) signaling pathway. Those include *RET/PTC* and *NTRK1* rearrangements and *RAS* and *BRAF* point mutations.

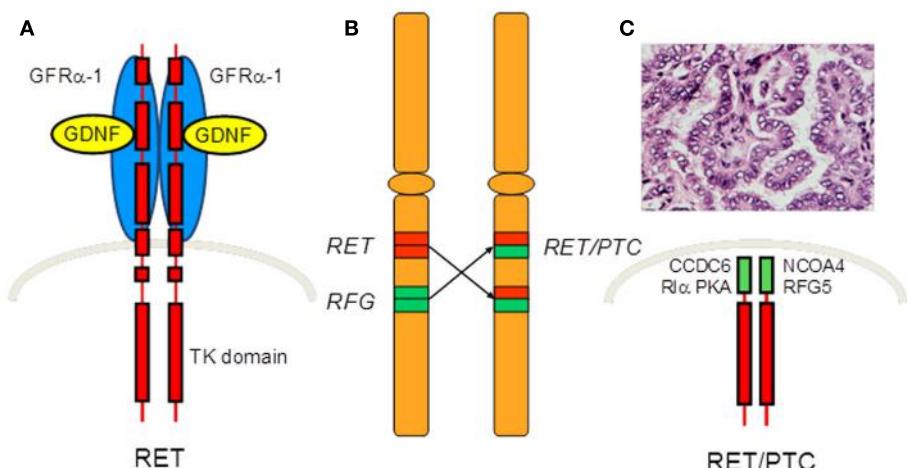


FIGURE 2 | Formation of RET/PTC. (A) *RET* codes a membrane protein of the tyrosine kinase receptor family which is not expressed or expressed at very low levels in thyroid follicular cells. (B) *RET* activation can occur through chromosomal rearrangements that involve the gene. (C) These

rearrangements result in the in-frame fusion of *RET* intracellular domain coding region with the 5' end of heterologous genes. The resulting chimeric sequence codes an oncoprotein, called *RET/PTC*, that drives the development of PTC.

In thyroid follicular cells, *RET* is not expressed or expressed at very low levels, but *RET* activation can occur through chromosomal rearrangements that involve the gene. They result in the in-frame fusion of part of *RET* intracellular domain coding region (including that coding for the TK from residue E713 and the carboxy-terminal tail) with the 5' end of heterologous genes (Figure 2). The resulting chimeric sequences are called *RET/PTC* and at least 13 variants have been reported to date, that differ according to the 5' partner gene involved in the rearrangement (Castellone and Santoro, 2008) (Figure 3). The two most

common rearrangement types are *RET/PTC1* and *RET/PTC3* which account alone for more than 90% of all rearrangements found in PTCs. *RET/PTC1* is formed by fusion with the coiled-coil domain containing gene 6 (*CCDC6*, formerly called *H4/D10S170*), and *RET/PTC3* by fusion with the nuclear receptor coactivator gene-4 (*NCOA4*, formerly called *RFG/ELE1/ARA70*; Grieco et al., 1990; Santoro et al., 1994). *RET/PTC1* and *RET/PTC3* are intra-chromosomal paracentric inversions because both genes participating in the fusion are located on chromosome 10q (Pierotti et al., 1992; Minoletti et al., 1994). At variance *RET/PTC2* and the other

rare types of *RET/PTC* are inter-chromosomal translocations (Castellone and Santoro, 2008).

Several mechanisms sustain the conversion of *RET* in a dominant oncogene for thyroid follicular cells after formation of *RET/PTCs* (Figure 4). In first instance, the ubiquitously expressed promoter of the fusion partner warrants ectopic *RET* expression in the cells harboring the rearrangement. Moreover, the partner genes are predicted to provide one or more coiled-coil domains that are essential for dimerization and ligand-independent activation of the truncated *RET* protein (Bongarzone et al., 1993; Tong et al., 1997; Monaco et al., 2001). Furthermore, *RET/PTCs* lack

the intracellular juxtamembrane domain which forms an integral part of the autoinhibited *RET* dimer interface (Knowles et al., 2006). As a consequence the TK domain is favored to adopt the active conformation. Finally, the delocalization of the rearranged protein to the cytosol is also expected to contribute to activation of the oncogene (Castellone and Santoro, 2008). In first instance, the altered subcellular localization uncouples *RET/PTCs* from receptor-mediated endocytosis and lysosomal degradation, leading to increased protein stability (Richardson et al., 2009). Moreover, it could prevent *RET/PTC* from interacting with negative regulators located at plasma membrane level, i.e., the tyrosine phosphatase Protein Tyrosine Phosphates Receptor type-J (PTPRJ; Iervolino et al., 2006).

The possible functional interactions between *RET/PTC* and EGFR in PCCL3 cells was examined after the observation that the epidermal growth factor receptor (EGFR) kinase inhibitor PKI166 decreased *RET/PTC* kinase auto-phosphorylation and activation of downstream effectors in thyroid cells (Croyle et al., 2008). This study showed that *RET/PTC* induces *EGFR* gene expression and kinase activation in part through MAPK signaling and forms a complex with EGFR in a kinase-independent manner. In turn EGFR stimulates *RET* phosphorylation (Croyle et al., 2008).

It is well known that PTC in general, but those harboring *RET/PTC* rearrangements in particular, present relatively indolent phenotypes compared with *RET*-related medullary thyroid carcinomas (MTC; Elisei et al., 2008). Considering that *RET/PTCs* possess similar if not greater oncogenic potential than membrane-bound *RET* when expressed at similar levels (Richardson et al., 2009), the explanation of this phenotype difference has to be searched in divergences of the oncogene expression levels or of the

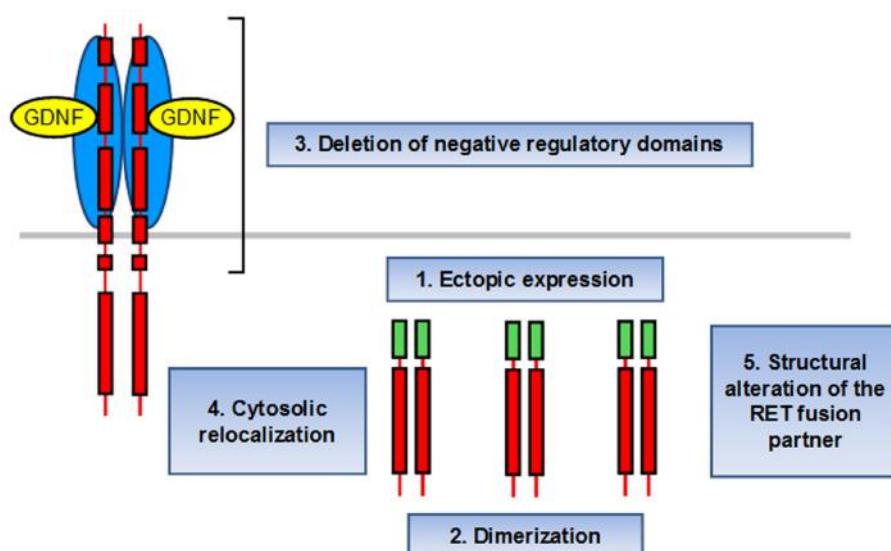
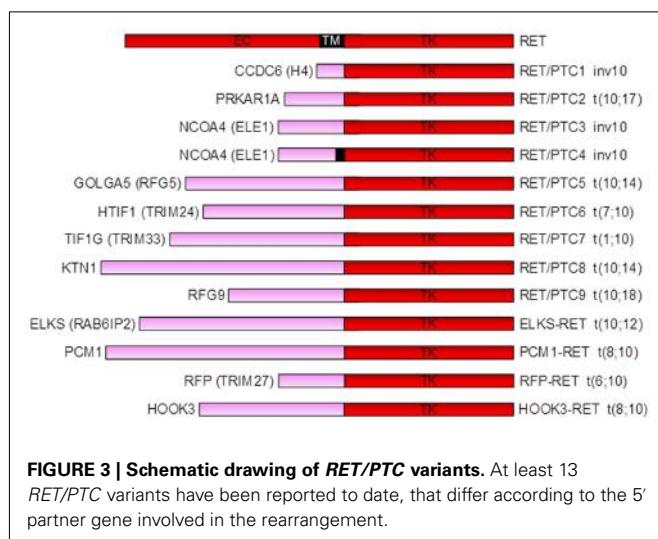


FIGURE 4 | Mechanism of *RET/PTC* activation. (1) The ubiquitously expressed promoter of the fusion partner warrants ectopic *RET* expression in the cells harboring the rearrangement. (2) The partner genes provide one or more coiled-coil domains that are essential for dimerization and ligand-independent activation of the truncated *RET* protein. (3) The lack of the inhibitory intracellular juxtamembrane domain favors *RET/PTC* to adopt the

active conformation. (4) The delocalization of the rearranged protein to the cytosol abolishes receptor-mediated endocytosis and lysosomal degradation, leading to increased protein stability, and interaction with negative regulators located at plasma membrane level. (5) *RET* fusion genes appear to code negative modulators of transformation. Thus, their structural alteration might contribute to the oncogenic potential of the *RET/PTC* rearrangements.

cellular responsiveness to them in follicular and the C-cells *in vivo*. Very recently, Richardson and others demonstrated that expression of RET/PTCs via the 5' promoter region of *CCDC6* and *NCOA4* could result in lower expression of these oncoproteins, relative to full-length RET, expressed off its endogenous promoter. These data indicate that also transcript level modulates the inherent oncogenicity of RET/PTC oncoproteins and that *in vivo* it is trumped by the substitution of relatively weak promoters upstream of the chimeric oncogene (Richardson et al., 2009).

It is plausible that also the structural alteration of the *RET* fusion partners might contribute to the oncogenic potential of the *RET/PTC* rearrangements. This is easily understandable for the *RET/PTC2* fusion partner *PRKARIA* which encodes a cyclic AMP-dependent protein kinase type I-regulatory subunit (Bongarzone et al., 1993). Indeed, it is known to be a bona fide tumor suppressor gene mutated in about half of Carney complex kindreds (Kirschner et al., 2000). Moreover, its ablation in the mouse induces the formation of thyroid tumors (Griffin et al., 2004). However, also *CCDC6* (*RET/PTC1*) has been shown to possess proapoptotic activity (Celetti et al., 2004) and to repress CREB1 transcriptional activity by recruiting histone deacetylase 1 and protein phosphatase 1 proteins at the CRE site of the CREB1

target genes (Leone et al., 2010). Moreover, *ERC1* (*ELKS-RET*) has appeared to be a *NF- κ B* regulator (Ducut Sigala et al., 2004; Wu et al., 2006), *TRIM33* (*RET/PTC7*) to interact with TGF β signaling intermediates Smad (Dupont et al., 2005; He et al., 2006), and *RFP* (*RFP-RET*) to act as a transcriptional repressor disabling pRb-mediated differentiation (Shimono et al., 2000; Krützfeldt et al., 2005).

Intriguingly, some of the *RET* fusion partners are found rearranged in tumors other than PTC: *TRIM24* (participating in *RET/PTC6*) rearranges with *BRAF* in mouse hepatocellular carcinomas (Zhong et al., 1999); *PMC1* (participating in *PMC1-RET*) with *JAK2* in chronic myeloid leukemia (Bousquet et al., 2005); *CCDC6* (participating in *RET/PTC1*) with the kinase domain of the platelet-derived growth factor receptor- β in myeloproliferative disorders (Kulkarni et al., 2000). Moreover, in thyroid carcinoma cell lines and PTC samples *CCDC6/H4* was shown to rearrange with *PTEN* through an intra-chromosomal inversion forming non-clonal *H4/PTEN* chimeric genes of unknown pathogenetic significance (Puxeddu et al., 2005b).

There are several lines of evidence pointing to *RET/PTC* as a key first step in thyroid cancer pathogenesis (Figure 5). In first instance, *RET/PTC* expression causes TSH-independent

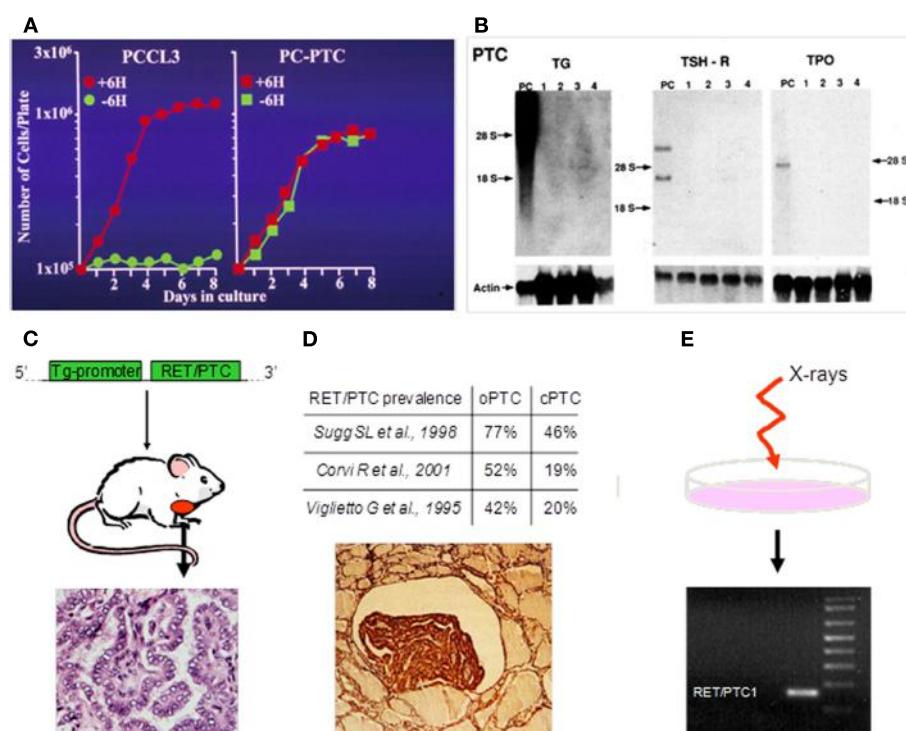


FIGURE 5 | Lines of evidence pointing to RET/PTC as a key first step in thyroid cancer pathogenesis. (A) RET/PTC expression causes TSH-independent proliferation in thyroid follicular cells. PCCL3: Parental well differentiated rat thyroid cell line. PC-PTC: PCCL3 cells stably expressing RET/PTC1. +6H: Grown in the presence of TSH. -6H: Grown in the absence of TSH (Modified from Santoro et al. (1993) with permission of the American Association for Cancer Research). (B) RET/PTC expression causes downregulation of differentiation markers in thyroid follicular cells. PC: Control parental PCCL3 cells. 1–4: Clones of PCCL3 cells stably expressing RET/PTC1. TG: Thyroglobulin. TSH-R: TSH receptor.

TPO: Thyroperoxidase. (From Santoro et al. (1993) with permission of the American Association for Cancer Research). (C) Thyroid-specific overexpression of either *RET/PTC1* or *RET/PTC3* in transgenic mice leads to development of tumors with histological features consistent with PTC (Jiang et al., 1996; Santoro et al., 1996; Powell et al., 1998). (D) Occult microscopic PTCs show high prevalence of *RET/PTC* expression (Viglietto et al., 1995; Sugg et al., 1998). (E) Exposure of cell lines and fetal thyroid explants to ionizing radiation, the major known risk factor for PTC, results in induction of *RET/PTC* expression within hours (Ito et al., 1993; Mizuno et al., 1997).

proliferation and downregulation of differentiation markers in thyroid follicular cells (Santoro et al., 1993). Moreover, thyroid-specific overexpression of either *RET/PTC1* (Jhiang et al., 1996; Santoro et al., 1996) or *RET/PTC3* (Powell et al., 1998) in transgenic mice leads to development of tumors with histological features consistent with PTC. Furthermore, there is a high prevalence of *RET/PTC* expression in occult microscopic PTC, believed to represent an early step in the development of clinically significant cancers (Viglietto et al., 1995; Sugg et al., 1998). Finally, exposure of cell lines (Ito et al., 1993) and fetal thyroid explants (Mizuno et al., 1997) to ionizing radiation results in induction of expression of *RET/PTC* within hours, supporting a direct role for radiation, the major known risk factor for thyroid cancer, in the illegitimate recombination of *RET*.

RET rearrangements have been considered exclusive of PTC. Unexpectedly, this dogma was broken very recently by three independent studies which simultaneously demonstrated the existence of a novel *RET* rearrangement in 1–2% of lung carcinomas. In this rearrangement, *RET* engages with *KIF5B* (the kinesin family 5B gene), located on chromosome 10p11.2, through a pericentric inversion of chromosome 10 (Kohno et al., 2011; Lipson et al., 2011; Takeuchi et al., 2011). Furthermore, one of these studies also reported the occurrence of *RET/PTC1* rearrangements in two lung adenocarcinomas (Takeuchi et al., 2011).

Four variants of *KIF5B-RET* fusion have been described and named on the basis of the last *KIF5B* and first *RET* exon involved in the fusion: K15;R12 (variant 1), K16;R12 (variant 2), K22;R12 (variant 3), K15;R11 (variant 4). All proteins encoded by the four *KIF5B-RET* rearrangements appeared to retain the *KIF5B* coiled-coil domain necessary for homodimerization and to hold the full *RET* kinase domain, as other types of oncogenic *RET* fusion proteins observed in PTC (Kohno et al., 2011; Lipson et al., 2011; Takeuchi et al., 2011).

The mutually exclusive nature of the *RET* rearrangements and other oncogenic alterations suggests that the *KIF5B-RET* fusion is a driver mutation in lung carcinoma (Kohno et al., 2011; Lipson et al., 2011).

KIF5B-RET expression in Ba/F3 cells led to oncogenic transformation, as demonstrated by the occurrence of interleukin-3 (IL-3)-independent growth. These transformed cells resulted sensitive to sunitinib, sorafenib, and vandetanib, which are all multitarget kinase inhibitors of *RET* (Lipson et al., 2011), suggesting that *RET* kinase inhibitors should be tested in prospective clinical trials in individuals with lung carcinomas that harbor *KIF5B-RET* rearrangements.

RET AND MEDULLARY THYROID CARCINOMA

Medullary thyroid carcinoma (MTC) is a malignant tumor arising from thyroid C cells. It represents about 5% of all thyroid cancers and can be hereditary (about 25% of cases) or sporadic (remaining cases; Stratakis and Ball, 2000). Familial forms develop in the context of Multiple Endocrine Neoplasia type 2 (MEN 2) syndrome in which MTC can be the sole manifestation of the disease (Familial Medullary Thyroid Carcinoma – FMTC) or be associated with pheochromocytoma (PC) and parathyroid hyperplasia/neoplasia (MEN 2A) or with PC, neuromas and body dimorphisms (MEN 2B). Germline mutations of the proto-oncogene *RET* confer

predisposition to all forms of familial MTC. The mutations fall into two main groups (Kouvaraki et al., 2005): (a) Those affecting the extracellular domain: These primarily involve cysteine residues 609, 611, 618 and 620 (exon 10), and 634 (exon 11). (b) Those affecting the RET-TK domain: These primarily involve codons 768, 790 and 791 (exon 13), 804 (exon 14), 883 and 891 (exon 15), and 918 (exon 16).

MEN 2A is associated mainly with germline mutations in the extracellular cysteine-rich region involving codons 609, 611, 618 and 620 (exon 10), and codon 634 (exon 11; 31–32). In particular, the Cys634Arg mutation specifically correlates with a higher risk of hyperparathyroidism (Mulligan et al., 1994). Rare mutations that are more commonly associated with MEN 2A include those at codons 768, 790 and 791 (exon 13), 804 (exon 14), and 891 (exon 15; Kouvaraki et al., 2005; Evans et al., 2007). MEN 2B is caused exclusively by mutations of residues within the intracellular TK domain of the receptor. Almost 95% of cases have a Met918Thr substitution (exon 16; Eng et al., 1996), and 3–4% are associated with Ala883Phe (exon 15; Gimm et al., 1997; Smith et al., 1997). Germline mutations in FMTC kindreds are more equally distributed throughout the *RET* gene and show a large overlap with those detected in MEN 2A (Kouvaraki et al., 2005; Evans et al., 2007).

All point mutations of *RET* in MEN 2 subjects are believed to have a “gain-of-function” effect resulting in unregulated activation of the TK activity of the receptor.

Mutations affecting cysteine residues in the extracellular domain are thought to result in ligand-independent receptor dimerization (Santoro et al., 1995; Carlomagno et al., 1997; Ito et al., 1997; Chappuis-Flament et al., 1998). It is likely that the cysteine residues normally form intramolecular disulfide bridges. Substitution of one partner cysteine by another aminoacid results in an unpaired cysteine, which is thought to link with its counterpart in an adjacent molecule. This event mimics conformational changes induced by ligand binding (Santoro et al., 1995). However, signaling of MEN 2A *RET* mutant is not identical to ligand-mediated *RET* signaling, as qualitative differences were identified, i.e., in the activation of the PI3K/AKT pathway (Frêche et al., 2005).

Mutations in the TK domain of *RET* constitutively activate the receptor without a requirement for dimerization. This phenomenon might be linked to the fact that some of the intracellular TK mutations target positions close to the trans-inhibited dimer contact points and may activate *RET* by destabilizing this inactive dimer conformation (Knowles et al., 2006). Moreover, the most frequent MEN 2B mutation (Met918Thr) was found to cause a great increase in ATP binding affinity and the formation of a more stable *RET*-ATP complex (Gujral et al., 2006). In addition to inducing “quantitative” changes of the basal kinase activity, the Met918Thr mutation is thought to affect also the “quality” of *RET*-generated intracellular signals by altering the affinity of the receptor to downstream substrates (Santoro et al., 1999).

Sporadic MTCs have no detectable germline abnormalities in *RET*. However, a significant proportion of these cancers has acquired a mutation in *RET* as a somatic event during the course of tumor initiation or progression (Romei et al., 1996; Elisei et al., 2008). Here, the mutation is present only in the tumor, and most often involves codon 918 (Met918Thr), although other

RET mutations have also been reported, such as some at codons 634 and 883. Estimates of prevalence of somatic *RET* mutations in sporadic MTC range from 23 to 70%. There are indications that tumors harboring *RET* mutations may carry a worse prognosis (Elisei et al., 2008).

The importance of *RET* mutations in the pathogenesis of either familial or sporadic MTCs has triggered the development of *RET* kinase inhibitors to be used in the clinical setting. At least four small molecules reached specific clinical trials in patients with unresectable, locally advanced or metastatic MTC: motesanib, vandetanib, cabozantinib, and lenvatinib (Puxeddu et al., 2011).

Motesanib was tested in a multicenter phase II study which enrolled 91 patients with advanced MTC (Schlumberger et al., 2009). The study yielded an objective response rate of only 2%, but a disease control rate (objective responses + stable disease) of 83%, with a median progression-free survival of 48 weeks and acceptable side effects. Compared to the very poor results of chemotherapy, these data appeared very promising. Unfortunately, further clinical development of motesanib was interrupted.

Vandetanib was tested in two phase II intervention studies (administering 300 and 100 mg per day, respectively) in patients with locally advanced or metastatic familial MTC (Robinson et al., 2010; Wells et al., 2010). Both studies showed objective responses in about 20% of subjects and stable disease in about another 50%, with no significant differences between the two dosages of the drug. In the 300 mg study median progression-free survival was longer than 27 months. An international, phase III, randomized, double-blinded, placebo-controlled, multicenter study to assess the efficacy of vandetanib vs. placebo in subjects with unresectable, locally advanced or metastatic MTC was recently completed (331 enrollments). The study met its primary objective of significant progression-free survival prolongation with vandetanib vs. placebo (22.6 vs 16.4 months. H.R. 0.45, 95% C.I. 0.30–0.69, $P = 0.0001$) (Wells et al., 2012), prompting FDA to approve the use of vandetanib to treat adult patients with late-stage (metastatic) MTC who are ineligible for surgery and who have disease that is growing or causing symptoms.

Cabozantinib was tested in a phase I dose-finding study which enrolled patients with various cancers, including an expansion cohort of 37 MTCs, showing some degree of tumor shrinkage in almost all patients of the latter group with 29% qualifying for a confirmed PR (Kurzrock et al., 2011). The good results obtained in the phase I study prompted the initiation of a registration phase III, randomized, double-blinded, placebo-controlled trial to determine the efficacy of the drug against placebo in advanced MTC patient, which is still ongoing². Similarly, clinical efficacy of lenvatinib in advanced MTCs is tested in an ongoing phase II study (see text footnote 2).

Interestingly, all these drugs are multikinase inhibitors whose mechanism of action does not rely only on targeting *RET*, but also VEGF receptors and receptors for other angiogenic factors (PDGFR β , c-KIT, c-MET) or growth factors (EGFR; Puxeddu et al., 2011). Vandetanib has been demonstrated, and the other considered TKI are expected, to inhibit also *RET/PTCs* and thus

to represent specific targeted therapies also for PTC harboring this class of gene rearrangements.

PREVALENCE OF *RET/PTC* REARRANGEMENTS

The prevalence of *RET/PTC* in PTCs varies significantly in different studies and geographic regions (Nikiforov, 2002). In the United States, the five largest series reported a frequency of *RET/PTC* ranging from 11 to 43% (Jhiang et al., 1992; Santoro et al., 1992; Lam et al., 1998; Tallini et al., 1998; Nikiforova et al., 2002), with totally 134 positive cases among 386 PTCs cumulatively studied (mean prevalence 35%). A comparable rate has been reported by other groups: Canada 40% (Sugg et al., 1999), Italy 29–35% (Santoro et al., 1992; Bongarzone et al., 1996, 1998). In other regions, a wide variation in frequency of *RET/PTC* has been reported, ranging from 3% in Saudi Arabia (Zou et al., 1994) to 85% in Australia (Learoyd et al., 1998). In most series, *RET/PTC1* is the most common type, comprising up to 60–70% of all rearrangements, whereas *RET/PTC3* accounts for 20–30% of positive cases and *RET/PTC2* for less than 10% (Nikiforov, 2002).

Several studies reported a higher prevalence of *RET/PTC* in PTCs from children and young adults (Bongarzone et al., 1996; Nikiforov et al., 1997; Soares et al., 1998; Fenton et al., 2000), indicating that young age predisposes to the formation of these mutations. Rapid thyroid cell proliferation may account for the particular high sensitivity of children's thyroids to chromosomal recombinations (Saad et al., 2006). In all studies *RET/PTC1* was the major type of rearrangement.

Finally, the prevalence of *RET/PTCs* appeared significantly higher in PTC from patients with a history of radiation exposure during childhood (Nikiforov, 2002). The Chernobyl nuclear power plant accident in April 1986 resulted in the release of large amounts of iodine isotopes, mainly ^{131}I , and, therefore, there was widespread exposure to the thyroid (Williams, 2002). A high incidence of childhood PTC was reported in contaminated areas (Figure 6). Among these PTCs, 67–87% of tumors removed 5–8 years after exposure and 49–65% of those removed 7–11 years after the accident harbored *RET/PTC* (Fugazzola et al., 1995; Nikiforov et al., 1997; Smida et al., 1999; Rabes et al., 2000). Interestingly, in post-Chernobyl tumors developed less than 10 years after the accident, *RET/PTC3* appeared to be the most common type, whereas those removed after the longer latency had predominantly *RET/PTC1* (Smida et al., 1999; Rabes et al., 2000). In patients subjected to therapeutic external irradiation during childhood the prevalence of *RET/PTC* was also high ranging between 54 and 84% (Bounacer et al., 1997; Elisei et al., 2001). In these cases *RET/PTC1* was more represented. However, exposure to ionizing radiations promotes the fusion of *RET* also with unusual partners and it is in this group that the majority of rare variants of *RET/PTCs* were detected (Nikiforov, 2002).

Geographic variation and differences in radiation exposure might partially explain the variability of *RET/PTC* frequencies in the different studies (Zhu et al., 2006). However, this cannot serve as the only explanation, because a striking variability in the frequency has been reported in the same geographical regions [8 and 85% in Australia (Learoyd et al., 1998; Chua et al., 2000), 5 and 77% in Canada (Sugg et al., 1996, 1998)].

²<http://clinicaltrials.gov/>

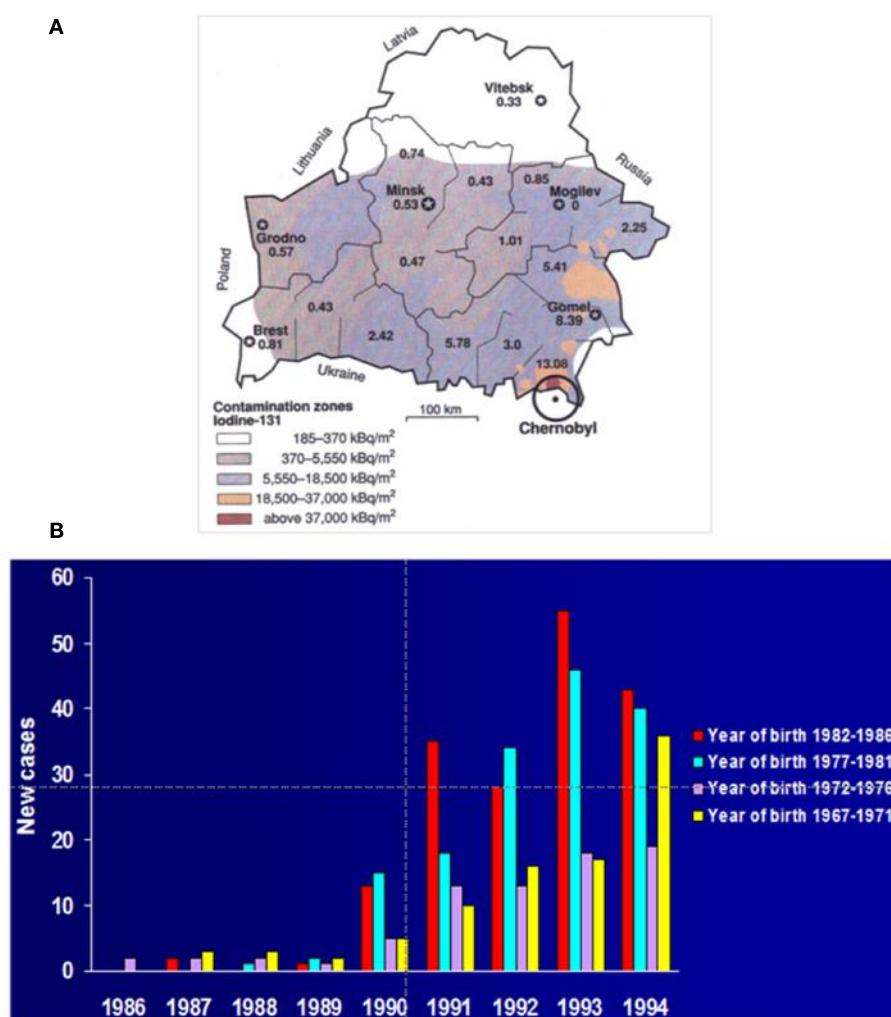


FIGURE 6 | Effects of the Chernobyl accident. (A) The Chernobyl nuclear power plant accident on April 26th 1986 resulted in the release of large amounts of iodine isotopes, mainly ¹³¹I, that contaminated the ground of surrounding regions, specially Belarus, with widespread exposure of the thyroids of the inhabitants. A high incidence of childhood PTC was reported in the more heavily contaminated areas (in the map small numbers indicate the incidence of pediatric thyroid cancers in the different

districts of Belarus, reported as number of cases per 100,000 children) [From Balter (1996) with permission of the American Association for the Advancement of Science]. **(B)** Unexpectedly, after the accident a rise of pediatric thyroid carcinomas was observed in Belarus with a latency as short as 4 years. The highest price was payed by the younger children at the time of the disaster [modified from Bleuer et al. (1997) with permission of Alphamed Press, INC.].

Thus, it is conceivable that the discrepancy between the reported prevalence of RET/PTC in PTCs may also be a result of different sensitivities of the detection methods and tumor genetic heterogeneity (Zhu et al., 2006). In the first instance, it has been clearly demonstrated that efficacy of RET/PTC detection, and of consequent reported prevalence, changed using various reverse transcriptase (RT)-PCR methods, or Southern blot, or fluorescence *in situ* hybridization (FISH). In the second instance, it has been clearly demonstrated that PTC can harbor RET/PTC rearrangement either as a clonal mutation, affecting the majority of tumor cells, or as a “non-clonal” one, affecting a small portion of tumor cells (Rhoden et al., 2006; Zhu et al., 2006). In the first case the genetic modification is likely to be important for tumor formation, in the second one the mutation

might represent only a casual alteration with no impact on the cancerogenetic process (“passenger mutation”). A counting of the second group in the RET/PTC-positive cases leads to an overestimation of the frequency of the mutation and to the inclusion of heterogeneous cases in the group of tumors driven by RET rearrangements.

The presence of RET/PTC rearrangements in non-neoplastic cells in Hashimoto’s thyroiditis is still controversial. It is possible that a heterogeneous presence of the rearrangement may account for such a controversy (Tallini and Asa, 2001; Nikiforov, 2002; Rhoden et al., 2006).

Tumor heterogeneity and multiclarity is also indicated by the presence of multiple RET/PTC variants in individual PTC samples (Sugg et al., 1998).

Among thyroid lesions, *RET/PTC* rearrangements have so far been identified only in PTC. They are absent in follicular adenomas and follicular thyroid carcinomas (Santoro et al., 2006).

In PTC, they are more frequent in the classic variant (Adeniran et al., 2006) and in microcarcinomas (Fusco et al., 2002), although they have been rarely described also in the cribriform variant (Cetta et al., 1998), in the Huerthle cell variant (Cheung et al., 2000; Chiappetta et al., 2002) and in hyalinizing trabecular adenomas (Cheung et al., 2000; Papotti et al., 2000). In radiation-induced PTC the solid variant is associated with the *RET/PTC3* oncogene, whereas the classic variant is associated with *RET/PTC1* (Nikiforov et al., 1997).

There is some controversy on the occurrence of *RET/PTC* rearrangements in poorly differentiated (PDTC) and anaplastic thyroid carcinomas (ATC) which are believed to derive in a significant number of cases from a stepwise dedifferentiation of well-differentiated PTCs and follicular thyroid carcinomas (Jhiang et al., 1992; Bongarzone et al., 1998; Soares et al., 1998; Tallini et al., 1998; Nikiforov, 2004). A recent study analyzed the mutational profile of advanced thyroid carcinomas of follicular origin, including primary PDTC and ATC and RAI refractory-FDG-PET positive-metastatic thyroid cancer lesions (Ricarte-Filho et al., 2009). It revealed that 18% of PDTC and 9% of RAI refractory-FDG-PET positive-PDTC harbored a *RET/PTC* rearrangement.

MECHANISMS OF *RET/PTC* FORMATION

As mentioned above, thyroid cancer, and in particular PTC, frequently harbor chromosomal rearrangements, including intra-chromosomal inversions and inter-chromosomal translocations. In all rearrangements, the formation of breaks in DNA strands must occur. There are various ways in which a cell can acquire these breaks, but exposure to ionizing radiation represents the best known mode (Weterings and Chen, 2008). DNA breaks are commonly repaired by two pathways, homologous recombination or non-homologous end joining (NHEJ; Shrivastav et al., 2008), and dysfunction of these pathways can contribute to the formation of chromosomal translocations (Gasparini et al., 2007). Alternatively, a fulsome accumulation of DNA breaks could prevent these normally functioning repair mechanisms, leading to translocation events.

Chromosomal fragile sites are regions of the genome that are prone to DNA breakage and are hotspots for chromosomal translocations. They are classified as common and rare, depending on their frequency in the population. Common fragile sites can be further classified based on their mode of induction, as not all sites are induced by the same compounds, or to the same extent. Aphidicolin (APH) induces expression of the majority of common fragile sites; other known fragile site-inducing conditions are the addition of 5-bromodeoxyuridine (BrdU), 5-azacytidine, and the removal of folic acid (Sutherland, 1991). Moreover, certain dietary and environmental factors have been shown to contribute to fragile site expression, including caffeine (Yunis and Soreng, 1984), ethanol (Kuwano and Kajii, 1987), hypoxia (Coquelle et al., 1998), and pesticides (Musio and Sbrana, 1997).

Fragile sites are also known to be late replicating regions of the genome. Delayed DNA replication has been observed in all fragile

sites examined to date (Handt et al., 2000; Hellman et al., 2000; Palakodeti et al., 2004; Pelliccia et al., 2008). Delayed replication at fragile sites is believed to be attributed to the high propensity of DNA to form stable secondary DNA structures (Gacy et al., 1995; Usdin and Woodford, 1995; Hewett et al., 1998; Mishmar et al., 1998; Zlotorynski et al., 2003; Zhang and Freudenreich, 2007).

Many studies point toward the association between fragile sites and formation of cancer-specific translocation (Arlt et al., 2006). Genes participating in the two main types of *RET/PTC* rearrangements, *RET/PTC1* and *RET/PTC3*, have been mapped to known fragile sites (Burrow et al., 2009). Both genes involved in the *RET/PTC3* rearrangement, *RET* and *NCOA4*, are located at 10q11.2 within fragile site FRA10G, a common fragile site induced by APH. The *CCDC6* gene, involved in *RET/PTC1*, is located at 10q21.2 within fragile site FRA10C, a common fragile site induced by BrdU. Major breakpoint cluster regions for these genes have been identified and are located within intron 11 of *RET*, intron 5 of *NCOA4* and intron 1 of *CCDC6* (Smanik et al., 1995; Nikiforov et al., 1999).

In a recent study, Gandhi and others showed the involvement of fragile sites in the formation of *RET/PTC* rearrangements. Using FISH and ligation-mediated PCR (LM-PCR) analysis, they provided structural and biochemical evidence that the *RET*, *CCDC6*, and *NCOA4* genes are located in common fragile sites FRA10C and FRA10G and undergo DNA breakage after exposure to fragile site-inducing chemicals. Moreover, exposure of human thyroid cells to these chemicals results in the formation of cancer-specific *RET/PTC* rearrangements (Gandhi et al., 2010). These results provide the direct evidence for the involvement of chromosomal fragile sites in the generation of cancer-specific rearrangements in human cells. In particular, activation of FRA10C and FRA10G might contribute to spontaneous formation of *RET/PTC1* and *RET/PTC3* rearrangements leading to the development of spontaneous non-radiation induced PTCs harboring the rearrangements.

Exposure to ionizing radiation is a well known risk factor for thyroid cancer, especially for PTC, which represents the most common form of radiation-induced solid neoplasm (Winship and Rosvoll, 1970; Ron et al., 1995). As reported above, a high prevalence of *RET/PTC* is seen in PTCs of individuals with a history of radiation exposure (Bounacer et al., 1997; Nikiforov et al., 1997; Rabes et al., 2000; Elisei et al., 2001). Moreover, among PTCs of survivors of the atomic bomb in Japan, the presence of *RET/PTC* directly correlated with the received radiation dose (Takahashi et al., 2007; Hamatani et al., 2008). The association between *RET/PTC* rearrangement formation and ionizing radiation is supported by several studies demonstrating the induction of *RET/PTC* by irradiation of cultured human thyroid cells (Ito et al., 1993; Caudill et al., 2005) and of human fetal thyroid tissue xenografts in SCID mice (Mizuno et al., 1997, 2000).

Ionizing radiation damages DNA in a variety of ways as a result of either direct energy transfer by radiation to DNA or by secondary reactive oxygen species produced by ionization of water. Of all types of DNA damage, double-strand breaks (DSBs) are considered a crucial primary lesion for a variety of biological end points, including cell killing, chromosomal aberrations, and cell transformation (Bryant and Riches, 1989; Winegar et al.,

1992). However, exactly how radiogenic DSBs lead to chromosomal rearrangements remains not fully understood. Several theories have been proposed but none of these can adequately explain the dose–effect relationship and complexity of radiation-induced aberrations (Edwards, 2002). The most widely accepted theory is the Breakage-and-Reunion theory. It postulates that chromosomal aberrations arise mainly as a result of the re-joining of two DSBs located closely in space and time (two-hit event; Savage, 1998; Hlatky et al., 2002). The initial distribution of primary breaks is thought to be random whereas the rejoining efficiency is expected to be influenced by their proximity (Yates and Morgan, 1993; Rothkamm et al., 2001).

It seems that nuclear architecture contributes to the generation of *RET/PTC* by placing potentially recombinogenic chromosomal loci in close proximity in the interphase nuclei of human cells. In a study that utilized FISH and three-dimensional (3D) confocal microscopy, it was demonstrated that *RET* and *CCDC6* genes, the partners of *RET/PTC1* rearrangement, were non-randomly located with respect to each other in the interphase nuclei of human thyroid cells and were much closer than expected based on their genomic separation (Nikiforova et al., 2000; **Figures 7A–C**). Moreover, this study showed that the proximity between potentially recombinogenic genes was cell-type specific and was present only in thyroid cells. In a more recent study, similar findings were obtained for *RET* and *NCOA4*, the partners of *RET/PTC3* rearrangement (Gandhi et al., 2006; **Figures 7D–E**).

In mammalian cells, DSBs are repaired by two pathways that are based on homologous or non-homologous recombination. The homology-dependent mechanism encompasses homologous recombination repair (HRR), single strand annealing (SSA), and non-allelic homologous recombination (NAHR). The non-homologous mechanism is known as non-homologous end joining (NHEJ). Another recently found repair pathway, micro-homology mediated end joining (MMEJ), combines features of the major pathways as it joins DNA ends after preliminary alignment using short homology DNA sequences located distant from the break.

Data obtained by the analysis of DNA sequences at the fusion points suggest that the formation of *RET/PTC* rearrangements may involve several possible DNA repair mechanisms, particularly NHEJ and MMEJ, and to lesser extent SSA (Bongarzone et al., 1997; Klugbauer et al., 2001). It remains unclear if all these pathways contribute to the generation of *RET/PTC* with similar frequency and if the choice is determined by specific conditions and/or individual genetic background.

Regardless of the specific DNA repair mechanism involved in recombination, spatial proximity is likely to predispose to specific rearrangements by making the neighboring regions prone to simultaneous damage by radiation or other DNA-damaging agents, and/or by facilitating mis-rejoining of free DNA ends located closely adjacent to each other (**Figure 7F**).

It remains unclear why specific chromosomal regions are located close to each other. For genetic loci located on the same chromosome, this is likely due to high order chromosome folding that would allow the genes to be positioned non-randomly with respect to each other. It has been found that the 18-Mb region on

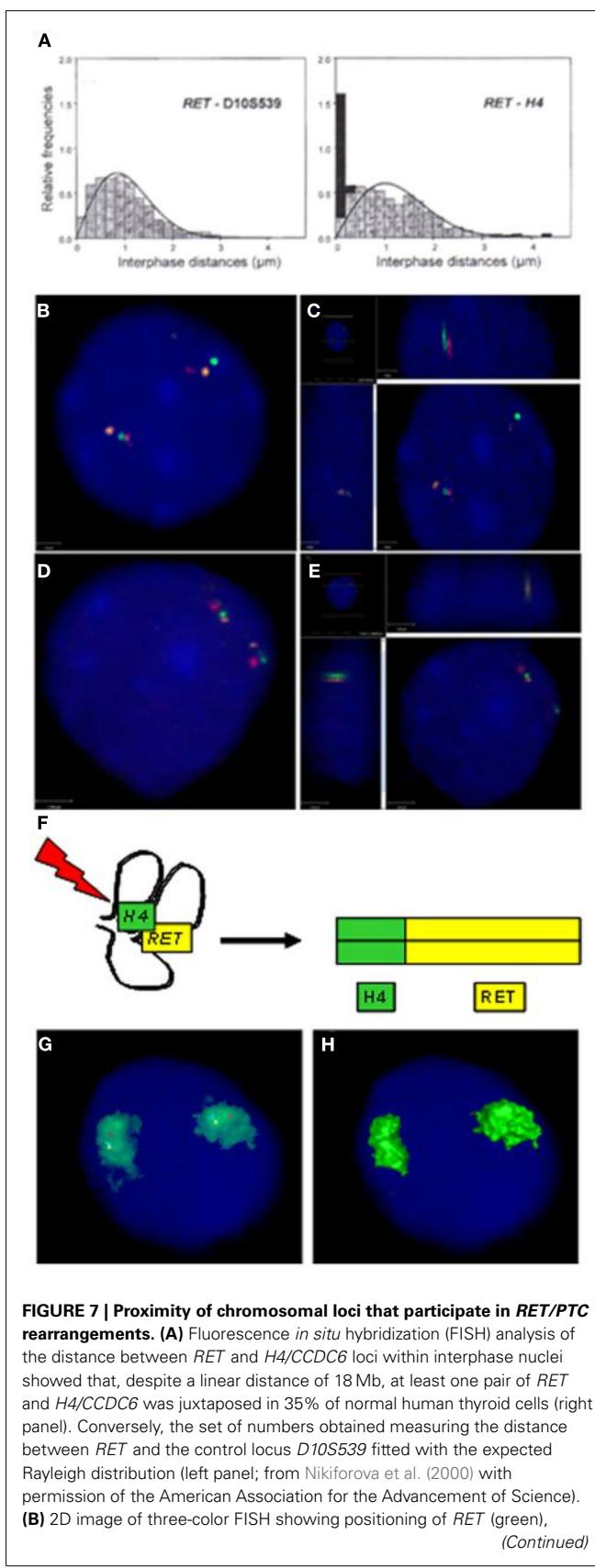


FIGURE 7 | Continued

NCOA4 (orange), and *H4/CCDC6* (red) in interphase nuclei of thyroid cells. The panel depicts a nucleus showing two sets of *RET*, *NCOA4*, and *H4/CCDC6* with one pair of *RET* and *H4/CCDC6* positioned close to each other. (C) 3D image showing that *RET* and *H4/CCDC6* are juxtaposed to each other in the same z plane. (D) 2D image of a nucleus showing one pair of *RET* and *NCOA4* positioned close to each other. (E) 3D image showing that *RET* and *NCOA4* are juxtaposed to each other in the same z plane (B–E are from Gandhi et al. (2010) with permission of Elsevier Ireland LTD). (F) Spatial contiguity of *RET* and the fusion partner gene may provide a structural basis for generation of *RET/PTC* rearrangement by allowing a single radiation track to produce a double-strand break in each gene at the same site in the nucleus (Nikiforova et al., 2000). (G) Four-color FISH showing chromosome 10 territory (green) and location of *RET* (blue pseudocolor), *NCOA4* (yellow pseudocolor), and *H4/CCDC6* (red). All three genes, *RET*, *NCOA4*, and *H4/CCDC6*, are positioned within the chromosome 10 territory (CT) and away from the CT edge. (H) 3D rendered image showing no signals on the surface of the CT due to the gene positioning inside the CT. (G–H are from Gandhi et al. (2010) with permission of Elsevier Ireland LTD).

10q containing *RET*, *NCOA4*, and *CCDC6* has a large-scale helical folding in the interphase nuclei of human thyroid cells. This pattern of chromatin folding can offer the basis for the proximity between *RET* and *NCOA4* and *CCDC6* (Gandhi et al., 2006).

A particular feature of rearrangements found in papillary thyroid cancers is that almost all of them are intra-chromosomal inversions rather than inter-chromosomal translocations; this may be due in part to the nuclear architecture. It was found that genes involved in intra-chromosomal rearrangements were positioned at significantly greater distances away from the chromosomal territory (CT) edge and internally within their CTs as compared to genes involved in translocations that were positioned closer to the CT edge (Gandhi et al., 2009). The frequent location of *RET* and its recombinogenic partners within the interior of CT and the limited availability of it to interact with neighboring chromosomal territories likely predispose it to intra-chromosomal inversions, such as that seen in most cases of *RET/PTC* (Figures 7G–H).

SIGNALING PATHWAYS ACTIVATED BY RET/PTC

Several RET/PTC-activated signal transduction pathways have been identified. In detail, RET/PTC signaling, as RET signaling, depends on the auto-phosphorylation of several tyrosine residues (Figure 8). Tyrosine (Y) 905 (numbered according to integral RET amino acid sequence) is a binding site for Grb7/10 adaptors whose function has not been completely elucidated (Wells and Santoro, 2009). Y905 is also a binding site for SH2B1 β , a protein that, by obstructing the SHP-1 tyrosine phosphatase, enhances RET phosphorylation (Donatello et al., 2007). Y1015 interacts with phospholipase C γ , thereby activating protein kinase C enzymes, which in turn are key regulators of receptor TKs (Andreozzi et al., 2003). Y1062 is a highly important multi-docking binding site for several signaling mediators such as DOK1/4/5, Enigma, FRS2, IRS1/2, Shc, and ShcC. Phosphorylation of tyrosine 1062 results in the activation of major intracellular mediators, including ERK/MAPK, PI3K-AKT, NF- κ B, and JNK (Wells and Santoro, 2009). Y1062-mediated MAPK activation appears to happen through the DOK4-Rap1 signaling cascade (Uchida et al., 2006; De Falco et al., 2007). Y1062 is essential for RET and RET/PTC transforming activity

(Segouffin-Cariou and Billaud, 2000; Knauf et al., 2003; Melillo et al., 2005). Y1096, present only in the RET51 splicing variant, binds Grb2 and appears to have a redundant behavior with Y1062 on the activation of the AKT/MAPK pathways (Jain et al., 2006).

Y752 and Y928 of MEN2A-RET appeared to be YxxV/Q Signal Transducer and Activator of Transcription 3 (STAT3) docking sites, involved in MEN2A-RET-induced phosphorylation of both Y705 and S727 residues of STAT3 (Schuringa et al., 2001). More recently, it was shown that RET/PTC1 and RET/PTC3 also interact with STAT3 and activate it through the phosphorylation of the tyrosine 705 residue (Hwang et al., 2003). However, RET/PTC1 residues Y141 and Y317, corresponding to RET residues Y752 and Y928, respectively, did not appear to be critical for the interaction with the transcription factor. Conversely, Y317 of RET/PTC1 appeared to be important for auto-phosphorylation of the kinase and for the Y705 phosphorylation of STAT3. This phosphorylation event appeared to require the intrinsic kinase activity of RET/PTC and to be independent from JAK2 and c-Src activation confirming that STAT3 is a direct substrate of RET/PTC in *vivo*. A physical interaction between RET/PTCs and the transcription factor could be demonstrated by co-immunoprecipitation experiments. Similarly, the expression of RET/PTC was found to lead to up-regulation of STAT1 expression and to the phosphorylation of the Y701 residue of this transcription factor (Hwang et al., 2004). Also in this case the dependence of the phosphorylation event from RET/PTC intrinsic kinase activity and its independence from JAKs and c-Src kinases activation pointed to the direct interaction between RET/PTCs and STAT1, although the occurrence of an association between RET/PTC and STAT1 could not be demonstrated by immune-precipitation analysis.

Among the non-phosphotyrosine mechanisms involved in RET/PTC signaling, binding of the PDZ-containing scaffold protein Shank3 to the C-tail of the RET9 splicing variant should be recalled. It contributes to sustained ERK and PI3K signaling (Schuetz et al., 2004).

Very recently, it was shown that RET/PTCs activate at least part of their pro-inflammatory programs through association with TRAF2 and TRAF6 which in turn activate NF- κ B by inhibiting the constitutive proteolytic degradation of NIK kinase. This pro-inflammatory signaling pathway appeared to be independent from PI3K/AKT and RAS/BRAF/MEK/ERK pathways. Thus, the possibility was proposed that RAS/BRAF and/or PI3K/AKT pathways are required for cellular transformation and that the additional pro-inflammatory pathways of RET/PTCs shape the features of the growing tumor (Wixted et al., 2011).

GENE EXPRESSION MODIFICATIONS INDUCED BY RET/PTCs IN CELLULAR MODELS

Gene expression modification induced by RET/PTCs has been studied principally and more consistently in cellular models.

A microarray analysis was conducted on well differentiated rat thyroid PCCL3 cells conditionally expressing the RET/PTC3 oncogene. Gene expression profiling 48 h after activation of RET/PTC3 identified a statistically significant modification of expression of 270 genes. Functional clustering of genes with a significant expression change revealed RET/PTC3-induced regulation of genes with key functions in apoptosis (*Ripk3*, *Tdga*), cell–cell signaling

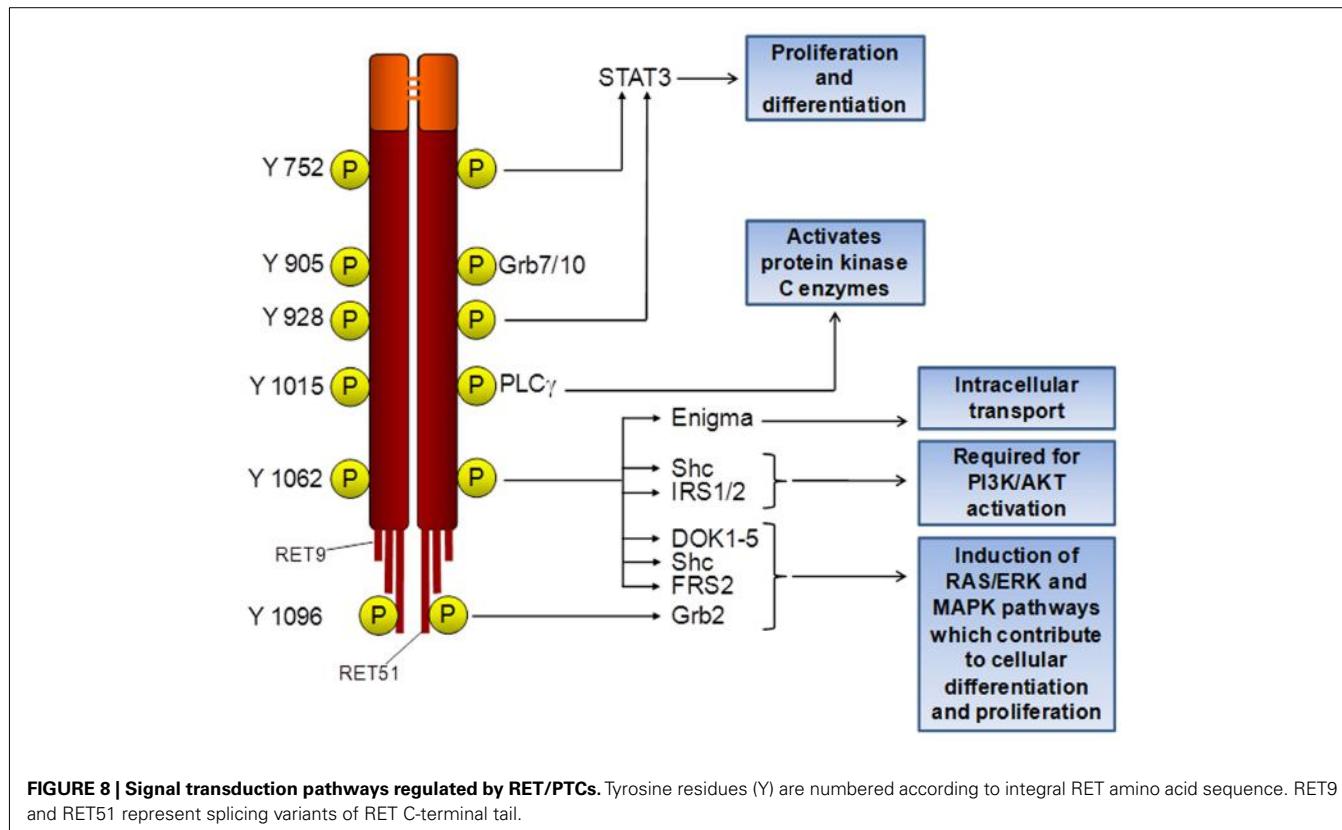


FIGURE 8 | Signal transduction pathways regulated by RET/PTCs. Tyrosine residues (Y) are numbered according to integral RET amino acid sequence. RET9 and RET51 represent splicing variants of RET C-terminal tail.

(*Cdh6*, *Fn1*), cell cycle (*Il24*), immune and inflammatory response (*Cxcl10*, *Scya2*, *Il6*, *Gbp2*, *Oas1*, *Tap1*, *RT1Aw2*, *C2ta*, *Irf1*, *Lmp2*, *Psme2*, *Prkr*), metabolism (*Aldob*, *Ptges*, *Nd2*, *Gss*, *Gstt1*), signal transduction (*Socs3*, *Nf1*, *Jak2*, *Cpg21*, *Dusp6*, *Socs1*, *Stat1*, *Stat3*, *Cish*), and transcription (*Nr4a1*, *Junb*, *Hfh1*, *Runx1*, *Foxe1*). This study clearly showed that genes coding for proteins involved in the immune response and in intracellular signal transduction pathways activated by cytokines and chemokines were strongly represented, indicating a critical role of RET/PTC3 in the activation and modulation of immune and inflammatory responses to the tumor (Figure 9) (Puxeddu et al., 2005a). The likely mediators of these expression clusters are NF- κ B, STAT1, and STAT3, based on data from the microarray and the literature (Hwang et al., 2003, 2004; Wixted et al., 2011) and preliminary data from our laboratory.

Similarly, exogenous expression of RET/PTC1 in primary normal human thyrocytes induced the expression of a set of genes involved in inflammatory/immune response including those encoding chemokines (*CCL2*, *CCL20*, *CXCL8*, and *CXCL12*), chemokine receptors (*CXCR4*) and cytokines (*IL1B*, *CSF-1*, *GM-CSF*, and *G-CSF*). Moreover, the modulation of genes involved in tumor invasion could be demonstrated, including matrix-degrading enzymes (metalloproteases and urokinase-type plasminogen activator and its receptor), and adhesion molecules (L-selectin). All these effects were strictly dependent on the presence of the RET/PTC1 Y451 residue (corresponding to RET Y1062 multidocking site). Selected relevant genes (*CCL20*, *CCL2*, *CXCL8*, *CXCR4*, *L-selectin*, *GM-CSF*, *IL1B*, *MMP9*, *UPA*, and

SPP1/OPN) were found up-regulated also in clinical samples of PTC, particularly those characterized by RET/PTC activation, local extrathyroid spread, and lymph node metastases (Borrello et al., 2005).

Another study analyzed the gene expression profile of the TPC1 cell line, derived from a papillary thyroid cancer lymph node metastasis and naturally harboring a RET/PTC1 rearrangement, and of the thyroid follicular cell line Nthy-ori 3-1 stably transduced with the oncogene. Among the genes found to be overexpressed, those involved in biological processes such as cell differentiation, proliferation, and cell signaling were over-represented. Such genes included *CEBPB*, *CCNG1*, *IFITM3*, *HTRA1*, *SEMA3F*, and *P300/CBP-associated factor* (PCAF). Among the genes shown to be down-regulated in RET/PTC1-expressing cell lines when compared to normal control, those involved in cell structure and motility, cytoskeletal remodeling, and cell signaling were over-represented. Examples of such genes included *DCTN5*, *TPM1*, *TPM3*, *CRP1*, Keratin type 1, Tropomyosin, *PSMD2* (TRAP2), and *RAB32*. Of particular interest in this study was the discovery of the under-expression of DROSHA in PTC cell lines. This is the core nuclease that executes the initiation step of miRNA processing in the nucleus. DROSHA collaborates with Dicer in stepwise processing of miRNAs and has a key role in miRNA mediated gene regulation processes such as development and differentiation. Interestingly, evaluation of the miRNA expression profile in the same cell lines revealed 21 overexpressed miRNAs and 14 underexpressed miRNAs in both cell lines when compared to Nthy-ori 3-1. Thus, miRNAs expression also appeared

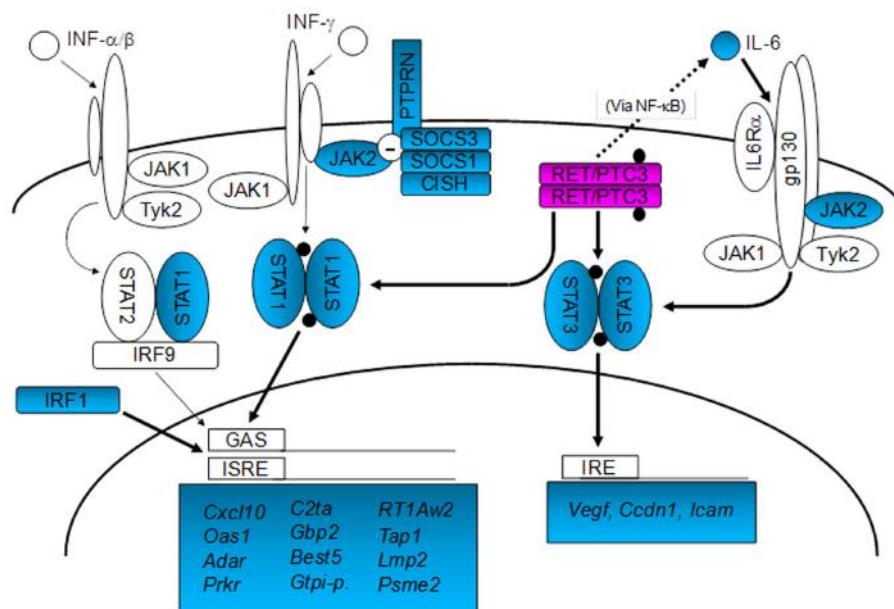


FIGURE 9 | Schematic map of the relationships between RET/PTC3 and IFN- and cytokine-activated signal transduction pathways according to the microarray results and to the literature. Up-regulated genes are shaded, whereas black dots indicate activation by phosphorylation. RET/PTC3 induces the expression of STAT1 and STAT3, involved respectively in the regulation of IFN and cytokine (e.g., IL-6) signal transduction pathways. RET/PTC3 also activates both transcription factors through direct phosphorylation of critical tyrosine residues (Hwang et al.,

2003, 2004) and through increased IL6 expression, possibly via NF- κ B (Wixted et al., 2011) (STAT3 activation). In addition RET/PTC3 induces the expression of additional components of these two pathways, such as JAK2, SOCS1 and 3, CISH and PTPRN. RET/PTC3-induced activation of STAT1 results in the up-regulation of IFN-responsive genes, whereas activation of STAT3 results in the up-regulation of VEGF, Cyclin D1, and ICAM (Hwang et al., 2003; modified from Puxeddu et al. (2005a) with permission of Bioscientifica Limited).

to be significantly affected by RET/PTC1 expression (Cahill et al., 2006).

Another study compared the transformed phenotype and the gene expression profile of rat thyroid PCCL3 cells stably expressing RET/PTC3 and its mutant RET/PTC3(Y1062F), BRAF(V600E), and H-RAS(V12). In the first instance, the authors could demonstrate that the oncogenic proteins involved in the initiation of PTC work along the same signaling cascade. This pathway starts at the level of RET tyrosine 1062 and sequentially triggers RAS, BRAF, and ERK stimulation. However, the analysis of transcriptional profiles indicated that the three oncogenes are not completely equivalent. Indeed, in addition to targets common to RET/PTC3, HRAS, and BRAF, there were relatively large sets of genes specifically modulated by only one or two of the three oncogenes. Many of the commonly modulated genes were involved in hallmarks of neoplastic transformation, e.g., altered cell morphology, uncontrolled growth, loss of differentiation, and apoptosis. RET/PTC3-induced genes included metalloproteinases (*Mmp3*, -10, -12, -13), those encoding adhesion/structure-associated proteins, such as collagen 1 (*Col1a1*), thymosin β 4, and galectin-3, and the dual-specificity phosphatase *Dusp6*. Moreover, the RET/PTC-RAS-BRAF signaling cascade appeared to stimulate the overexpression of some chemokines/cytokines (*Cxcl1*, *Cxcl10*, and *Ccl2*). Thus, this study too confirmed a strict relationship between thyroid cancer and inflammation in which tumors appear to use molecules of the innate immune system not only to recruit leukocytes, but also for growth, survival, and metastasis (Melillo et al., 2005).

A final study which evaluated RET/PTC3-induced gene expression profile in a cellular model was a follow-up study of the first one described in this paragraph (Puxeddu et al., 2005a). It compared PCCL3 cells with conditional expression of RET/PTC3 to PCCL3 cells with conditional expression of BRAF(V600E) or RET/PTC3 in the presence of small interfering RNA-mediated knockdown of BRAF. Among the RET/PTC3-induced genes, 2,552 did not require BRAF as they were similarly regulated by RET/PTC3 with or without BRAF knockdown and not by expression of BRAF(V600E). Acquired immune response and IFN-related genes were highly represented in this group. Conversely, about 24% of RET/PTC3-regulated genes were BRAF dependent, as they were similarly modified by RET/PTC3 and BRAF(V600E) but not in cells expressing RET/PTC3 with knockdown of BRAF. A significant subset of genes involved in innate immune responses belong to this group. Among them were the monocyte-macrophage chemoattractants *Mcp1* (*Ccl2*), *Mcp3*, *Gm-Csf*, and *Ccl15*. Moreover, a gene cluster coding for components of the mitochondrial electron transport chain pathway was down-regulated in this group, potentially altering regulation of cell viability. Genes coding for metalloproteinases were also preferentially induced by BRAF, particularly *Mmp3*, *Mmp9*, and *Mmp13*. This study recapitulated most of the data reported by the previous ones indicating a fairly clear demarcation between some of the functional gene programs activated by RET/PTC3 and BRAF in thyroid cells. RET/PTC3 appeared to induce expression of a remarkably rich array of genes involved in acquired immunity, whereas BRAF appeared to evoke

expression of genes that promote activation of an innate immune reaction. Moreover, although both genes induced expression of genes involved in matrix remodeling, RET/PTC did so less robustly, which could account in part for the lower predisposition of PTC harboring *RET/PTC* rearrangements to invade surrounding tissues compared to those harboring *BRAF* mutations (Mesa et al., 2006).

INSIGHTS ON RET/PTC INFLUENCES ON TUMOR MICROENVIRONMENT

RET/PTC oncoproteins are believed to take part in several mechanisms that allow tumor growth and spread, including angiogenesis, invasion, metastasis, and immune escape. RET/PTC induces these significant phenotypic changes oriented toward neoplastic transformation affecting the tumor microenvironment.

Eicosanoids are thought to play an important role in survival, growth and metabolic support of tumor cells (Koki et al., 2002). Significant among them are prostaglandins, of which prostaglandin E₂ (PGE₂) is the most abundant in nature. The synthesis of PGE₂ from arachidonic acid requires two enzymes acting sequentially. The first step is catalyzed by cyclooxygenase (COX), which transforms arachidonic acid in the endoperoxide prostaglandin H₂ (PGH₂). The second step is catalyzed by prostaglandin E synthase (PGES), which converts PGH₂ in PGE₂. There are two COX isoforms: COX-1 and COX-2. COX-1 is constitutively expressed in most tissues whereas COX-2 is normally not expressed but is induced by growth factors, cytokines and certain oncogenes (Smith and Langenbach, 2001). A variety of epithelial cancers have increased expression of COX-2, including colorectal (Eberhart et al., 1994; Yoshimatsu et al., 2001) and thyroid carcinomas (Nose et al., 2002; Specht et al., 2002). There are three PGES isoforms: cytosolic PGES (cPGES), microsomal PGES-1 (mPGES-1) and mPGES-2. cPGES is expressed constitutively and has been proposed to exhibit preferential functional coupling with COX-1 (Tanioka et al., 2000). mPGES-1 is induced by pro-inflammatory stimuli and increases during the period when COX-2-dependent PGE₂ generation is ongoing (Murakami et al., 2000; Mancini et al., 2001). mPGES-2 does not show homology with mPGES-1 (Tanikawa et al., 2002) and has been proposed to couple functionally to both COX-1 and COX-2 (Murakami et al., 2003). mPGES-1 is overexpressed in most colorectal adenomas and carcinomas, suggesting that this may contribute to the increased amounts of PGE₂ in these tumors.

Conditional expression of *RET/PTC1* or *RET/PTC3* in PCCL3 thyroid cells markedly induced both COX-2 and mPGES-1 mRNA and protein. Accordingly, conditioned media of these cells, analyzed by HPLC or ELISA, showed a significant increase of PGE₂ concentration (Figure 10). Based on signal transduction dissection experiments, RET/PTC was found to regulate mPGES-1 expression through the MAPK pathway (Puxeddu et al., 2003a). These data indicate a direct relationship between RET/PTC activation and regulation of PGE₂ synthesis. It can be speculated that paracrine action of PGE₂ might influence tumor development and progression regulating angiogenesis and the anti-tumor immune response. However, further investigation is needed to fully understand these phenomena.

It has been hypothesized that the *RET/PTC* oncogene contributes to tumor invasion. Castellone and others, explored the transcriptional response of PCCL3 cells to *RET/PTC* oncogene expression looking for the overexpression of genes involved in tumor cell diffusion. They found that among several genes induced by RET/PTC, CXCR4 showed approximately a threefold increase. Western blot with anti-CXCR4 polyclonal antibody confirmed the overexpression of CXCR4 in rat transformed thyroid cells (Castellone et al., 2004). CXCR4 is the receptor for the chemokine CXCL12/SDF-1 α/β . Chemokines are chemoattractant cytokines that play a major role in the recruitment of leukocytes to sites of inflammation. They are secreted in the tumor microenvironment by infiltrating inflammatory cells and by tumor cells (Balkwill and Mantovani, 2001; Coussens and Werb, 2002). Chemokines bind to seven-transmembrane receptors present on the cell surface that are coupled to G proteins, such as CXCR4, and activate a cascade of cellular events that result in cell polarization, adhesion, and migration (Melillo et al., 2005). Castellone and others observed that CXCR4 expression correlated with the integrity of RET/PTC catalytic domain and depended on the activation of the RET/PTC-RAS-ERK signaling pathway. They also found that CXCR4 was expressed in *RET/PTC*-positive human thyroid cancer cells but not in normal thyroid cells. Treatment with SDF-1 α caused an increase of the proliferation and survival of PCCL3 cells expressing *RET/PTC1* (PC-PTC1 cells) and of Matrigel invasion of PC-PTC1 and human FB2 cells (PTC-derived cell line expressing *RET/PTC1*; Castellone et al., 2004). Taken together, these results suggest that human thyroid cancers harboring *RET/PTC* rearrangements may use the CXCR4/SDF-1 α receptor-ligand pathway to proliferate, survive, and migrate.

Moreover, there are other reasons why the expression of CXCR4 in human PTCs could be important. In the first instance, it might contribute to the preferential localization of PTC metastases to lymph nodes. Indeed, lymph nodes have been indicated as sites of high production of SDF-1 α (Müller et al., 2001). Moreover, CXCR4 expression might enhance the tumor inflammatory infiltrate through the activation of signal transduction pathways that lead to the secretion of cytokines. Indeed, it has been shown that stimulation of CXCR4 in ovarian cancer leads to the production of TNF- α , which, in turn, can behave as a growth factor for cancer cells or mediate other events such as recruitment of leukocytes to the tumor site (Scotton et al., 2002).

As already reported above, Melillo showed that *RET/PTC3*, *HRAS(V12)*, and *BRAF(V600E)* oncogenes activate a common transcriptional program in thyroid cells that includes CXCL1/growth-related oncogene- α (CXCL1/GRO- α) and CXCL10/interferon- γ -inducible protein 10 (CXCL10/IP-10) chemokines. According to the microarray results, QPCR data demonstrated that CXCL1 and CXCL10 were up-regulated also in human PTCs. CXCR2 (the CXCL1 receptor) and CXCR3 (the CXCL10 receptor) were expressed in parental and transformed PCCL3 cells and up-regulated in all the PTC cell lines examined in comparison to normal thyroid follicular cells. Treatment of TPC1 cells with recombinant CXCL1 and CXCL10 stimulated DNA synthesis and cell invasiveness through Matrigel (Melillo et al., 2005).

These data are very interesting in light of the fact that at least 30% of PTC present a chronic inflammatory reaction (Rosai et al.,

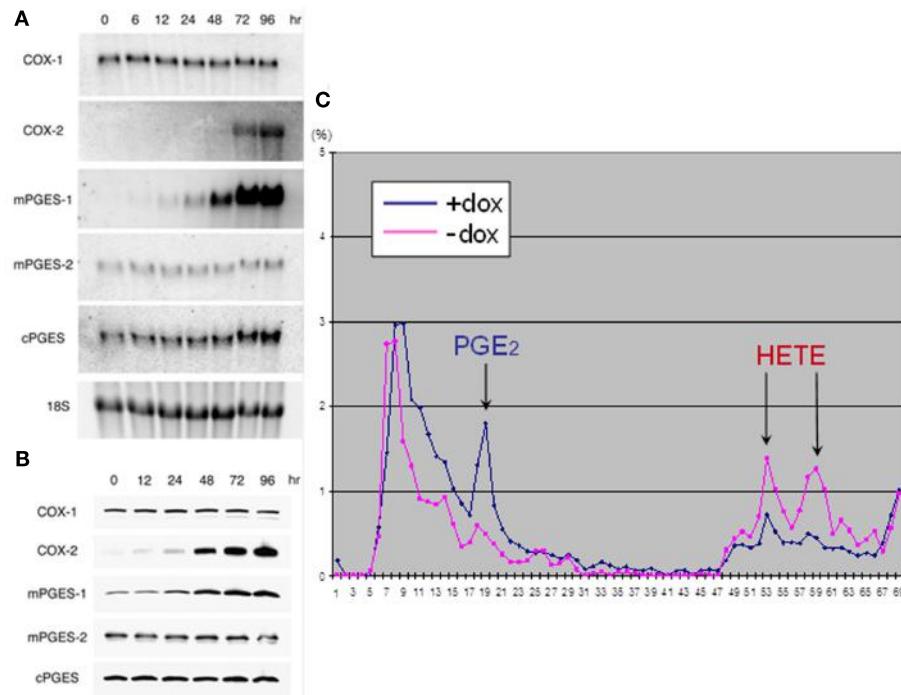


FIGURE 10 | RET/PTC3 regulates PGE₂ biosynthesis in PCCL3 cells characterized by the doxycycline (DOX)-inducible expression of the oncogene (PTC3-5 cells). (A) Northern blots showing that treatment with DOX for up to 96 h induces COX-2 and mPGES-1 mRNA but not COX-1, mPGES-2, or cPGES. **(B)** Western blots confirming that activation of

RET/PTC3 expression for up to 96 h induces COX-2 and mPGES-1 protein but not COX-1, mPGES-2, or cPGES. **(C)** HPLC analysis of conditioned medium of PTC3-5 cells metabolically labeled with [³H]arachidonic acid showing that doxycycline (DOX) treatment induces PGE₂ synthesis and secretion in the medium [modified from Puxeddu et al. (2003a)].

1992; Scarpino et al., 2000). A molecular explanation of this phenomenon could be the fact that the RET/PTC-RAS-BRAF signaling cascade stimulates the overexpression of several chemokines. In turn, chemokines secreted by cancer cells can recruit leukocytes to the tumor site (Luster, 1998; Mellado et al., 2001). Moreover, it can be envisaged that these chemokines secreted by the cancer cells in the tumor microenvironment can act in an autocrine/paracrine fashion to support proliferation and cell motility of cancer cells themselves.

By using PCCL3 cells, osteopontin (OPN) has been identified as a major transcriptional target of RET/PTC in thyroid cells. OPN, also known as SPP1 (secreted phosphoprotein 1) is a highly acidic calcium-binding glycosylated phosphoprotein that binds to the cell surface receptors α_v - or β_1 -containing integrins and CD44 (Weber, 2001). It is also regarded as a cytokine (ETA-1, Early T-lymphocyte antigen, or IL-28) regulating T helper cell-1 function (Ashkar et al., 2000; Chabas et al., 2001). OPN supports several functions including chemotaxis, cell attachment, and cell migration; involvement of OPN in tumorigenesis and metastasis formation has elicited much interest (Weber, 2001).

CD44 is a cell surface glycoprotein that can be expressed as a standard receptor (CD44s) or as multiple splice isoforms (CD44v) whose expression is altered during tumor growth and progression. Expression of the v6 exon variant is necessary for efficient OPN binding (Weber, 2001; Ponta et al., 2003). Normally, only CD44s is expressed on the cell surface of non-proliferating

thyrocytes, whereas CD44v6 is invariably overexpressed in PTC samples (Ermak et al., 1996; Bartolazzi et al., 2001). CD44 has been implicated in cell-cell and cell-matrix interactions and homing of tumor cell metastasis (Ponta et al., 2003).

OPN is expressed in several human tumors, such as colon, breast, prostate, ovarian, gastric, and lung carcinomas. Moreover, OPN expression often correlates with a poor prognosis (Rittling and Chambers, 2004). It has been shown that OPN produced by RET/PTC-transformed thyrocytes cooperates with RET/PTC to increase DNA synthesis and invasiveness of PCCL3 cells (Castellone et al., 2004).

In a more recent work, OPN and CD44 expression were evaluated by immunohistochemistry with specific monoclonal antibodies. It has been found that the prevalence and intensity of OPN staining significantly correlated with the presence of lymph node metastases and tumor size. It has also been shown that treatment of human PTC cells with recombinant exogenous OPN stimulated Matrigel invasion and activated the ERK and AKT/PKB signaling pathways (Guarino et al., 2005).

Because OPN and CD44v6 overexpression is a common feature not only of PTC cells that harbor RET/PTC but also of those expressing BRAF(V600E), the activation of the OPN-CD44v6 axis appears as one of the end points of the RET-RAS-BRAF-MAPK oncogenic cascade. This model is consistent with the idea that OPN expression is also induced by other oncogenes such as RAS and is dependent on the MAPK pathway and that CD44 splicing

is under control of the RAS-MAPK pathway as well (Geissinger et al., 2002; Matter et al., 2002).

The association between lymphocytic infiltrates and thyroid cancer, particularly PTC, in humans is also well documented (Dailey et al., 1955; Matsubayashi et al., 1995).

The observation that lymphocytic infiltration of the thyroid coexists with cancer suggests that antigens expressed by tumor cells are responsible for inducing an acquired anti-tumor immune response (Segal et al., 1985). The study of immune responses to tumors has primarily focused on the identification of tumor-associated antigens capable of serving as antigenic targets of anti-tumor immune responses (Van den Eynde et al., 1991; Rosenberg, 1997). Despite the presence of a unique tumor antigen and demonstration of an anti-tumor T cell response, cancer often persists in mouse tumor models as well as in cancer patients (Lee et al., 1999; Yu et al., 2005). These observations led to a new vision of inflammatory cells as a means of tumor support and a necessity for cancer progression. Inflammatory cells provide cytokines, growth factors, proangiogenic mediators, and activating anti-apoptotic pathways that supply a supportive environment for cancer cell survival and tumor development (Balkwill et al., 2005; de Visser et al., 2006). These cytokines and growth factors also promote recruitment and establishment of non-transformed host-derived fibroblast and endothelial cells, creating a supportive framework for cancer cells (Moore et al., 1999; Olumi et al., 1999). Moreover, chronically activated inflammatory cells within tumors can play a protective role in suppressing adaptive anti-tumor responses (Zou, 2005).

The study of the properties of *RET/PTC3* has revealed dual immunological features. First, its constitutive activation can induce the nuclear transactivation of the NF- κ B protein complex (Visconti et al., 1997; Hayashi et al., 2000; Ludwig et al., 2001), driving the expression of several pro-inflammatory mediators (Ghosh and Karin, 2002; Puxeddu et al., 2005a). Second, *RET/PTC3* encodes a chimeric protein that includes peptides that may be targets of anti-tumor immune responses (Viglietto et al., 1995; Fusco et al., 2002).

Pufnock and Rothstein hypothesized that combined immune and neoplastic properties of *RET/PTC3* play a role in tumor progression and that these properties are dependent on the immune system. They studied the immunological functions of *RET/PTC3* in an *in vivo* cancer model by measuring the recruitment of inflammatory cells in the tumor microenvironment and the effects on tumor growth. They found that tumor take in immunocompetent mice was more frequent using *RET/PTC3* tumor tissue rather than the control *RET/PTC^{Y588F}* one (mutation of tyrosine 588 eliminates the transforming ability of *RET/PTC3*). *RET/PTC3* expression correlated with increased CD8 $^{+}$ T cell recruitment at an early stage of tumor progression and induced recruitment of myeloid-derived CD11b $^{+}$ Gr1 $^{+}$ cells into tumors. Thus, tumors expressing the functional form of *RET/PTC3* showed enhanced infiltration of CD8 $^{+}$ lymphocytes, myeloid-derived CD11b $^{+}$ Gr1 $^{+}$ cells and enhanced growth in immunocompetent mice. In contrast, *RET/PTC3* signaling mutant-expressing tumors maintained enhanced infiltration of CD8 $^{+}$ lymphocytes but showed a lower recruitment of CD11b $^{+}$ Gr1 $^{+}$ cells and a decreased tumor incidence. Pufnock and Rothstein (2009) proposed that

enhanced transactivation of NF- κ B by *RET/PTC3* increases the quantity of secreted cytokines, thereby increasing the number of myeloid-derived CD11b $^{+}$ Gr1 $^{+}$ cells recruited into tumor tissue.

Other studies showed that CD11b $^{+}$ Gr1 $^{+}$ cells had strong suppressive activity on CD8 $^{+}$ tumor-specific responses (Serafini et al., 2004). An increase in CD11b $^{+}$ Gr1 $^{+}$ cells in *RET/PTC3*-expressing tumors at an early stage of tumor progression may account for both the enhanced tumor incidence and larger tumor size observed despite the high number of CD8 $^{+}$ cells in the tumors.

Recently, it was demonstrated that IFN- γ , derived from intratumoral-activated T cells, was necessary to activate the suppressive function of CD11b $^{+}$ Gr1 $^{+}$ cells (Gallina et al., 2006). These data confirm the notion that the increase of T cell activation at the tumor site, when combined with localization of myeloid-derived CD11b $^{+}$ Gr1 $^{+}$ cells, may promote an environment supporting cancer progression rather than regression, in the contest of a potential anti-tumor response. Although Pufnock and Rothstein suggested that immature CD11b $^{+}$ Gr1 $^{+}$ cells act in a suppressive manner on CD8 $^{+}$ cells, further evaluations are necessary to determine whether this interaction provides direct help to growing tumors through the secretion of growth factors rather than, or in addition to, suppression of directed anti-tumor CD8 $^{+}$ cytolytic responses. At any rate, these data suggest that *RET/PTC3* enzymatic functions might independently regulate cellular transformation and anti-tumor lymphocytic responses promoting tumor progression from an early stage. The immunological functions seem to influence both innate and adaptive immune cells present at the site of the growing tumor. Furthermore, *RET/PTC3*-induced NF- κ B activation may be the key pathway governing the immunomodulatory functions of the oncoprotein.

CD8 $^{+}$ T cell infiltration of the tumors has been hypothesized to represent an oncoprotein-reactive lymphocytic accumulation. The immunogenic capacity of *RET/PTC3* fusion protein has been explored. It was demonstrated that the fusion oncoprotein behaves like an antigenic non-self protein and a thyroid-specific tumor antigen. Although *RET/PTC3* is the fusion of two self proteins to which immunological tolerance is most likely induced, it was found that the fusion modifies the immunological properties of the molecules. Interestingly, it was detected that the immunogenic response to *RET/PTC3* was not directed against the peptide comprising the unique fusion region but rather against the carboxyl-terminal portion of *RET/PTC3* that derives from the self protein c-RET. Furthermore, transplantation of *RET/PTC3*-expressing thyroid cancers into naïve mice resulted in leukocytic infiltration, tumor rejection and induction of *RET/PTC3*-specific T cells (Powell et al., 2003). Thus, the somatic fusion of two unrelated self proteins appears able to trigger a uniquely immunogenic response directed against self epitopes within *RET/PTC3*.

In summary, *RET/PTC* appears to activate innate and acquired immunity in several ways. In a first phase, these processes might be instrumental to tumor elimination or to instauration of equilibrium between the tumor and the immune system. However, with time the cancer cell develops progressive immune resistance and gains the ability to tilt the immune response from restraining to advantageous for the tumor growth. The iteration of evasion mechanisms, associated with the suppression of different components of the immune system, results finally in

immune escape. Moreover, several humoral and cellular components of the immune system itself may start to contribute to cancer maintenance and progression.

DOES RET/PTC IMPACT ON THE PROGNOSTIC STRATIFICATION OF THYROID CARCINOMA?

Considering the above-described features of RET/PTCs and their transforming mechanisms, the question of whether *RET/PTC* has a role as a thyroid carcinoma tumor marker arises. Regarding thyroid cancer diagnostics the answer is yes, and because this issue goes beyond the aims of this review, we refer to a very recent work on this topic (Nikiforov, 2011). Regarding prognostic stratification, the problem appears to still not be solved. Because of the lack of extensive reviews on this problem, we dedicate this paragraph to review the impact of *RET/PTC* on the prognostic stratification of thyroid carcinoma.

Several studies have tried to associate the presence of a rearranged *RET* with clinical parameters. Unfortunately, the results obtained are controversial. Some studies indicated an association of *RET/PTC* with a poor prognosis. Conversely, others indicated an association with a good prognosis. Finally, a last group could not find any association with prognosis at all.

In post-Chernobyl cases, rearrangement-positive PTCs appeared in a more advanced pT category and more frequently in the pN1 category at presentation than rearrangement-negative PTCs (Rabes et al., 2000). Moreover, *RET/PTC3*, the dominant rearrangement in the post-Chernobyl PTCs presenting with short latency, was related to the solid variant of PTC, considered by some researchers as a more malignant histotype (Thomas et al., 1999; Rabes et al., 2000). Accordingly, also in sporadic PTCs it was suggested that the rearrangement of the *RET* proto-oncogene may be involved in the development of local invasion (pT4) and distant metastases (Jhiang et al., 1992; Mayr et al., 1997; Bongarzone et al., 1998).

Other studies indicated the exact opposite. Viglietto and co-workers showed a higher frequency of *RET/PTC* rearrangements in papillary thyroid microcarcinomas compared to clinically significant PTCs (42 vs 20%; Viglietto et al., 1995). Soares et al. (1998) showed the occurrence of the rearrangement in well differentiated slow growing neoplasms characterized in their view by a “Bonsai” phenotype. Finally, Tallini et al. (1998) confirmed the association of *RET/PTC* rearrangements with well differentiated or undercentimetric PTCs and showed that in their hands the subset of *RET/PTC*-positive PTCs do not progress to more aggressive, less differentiated tumor phenotypes.

Finally, at least three studies did not show any correlation between *RET/PTC* activation and age, sex, tumor size, TNM staging, number of neoplastic foci, and histological subtype (Learoyd et al., 1998; Elisei et al., 2001; Puxeddu et al., 2003b).

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It is likely that these studies have all been impaired by the lack of a unique and validated technique to detect *RET/PTC* rearrangements (see “Prevalence of *RET/PTC* Rearrangements”). Moreover, most of them are characterized by a far too small size to arrive to any consistent conclusion. However, probably with the exception of radiation-induced PTCs in which *RET/PTC* might define a subset of more aggressive neoplasms, some evidence points to a good prognostic impact of the rearrangements compared to other genetic alterations. In the first instance, it is clear that PTC harboring *BRAF* mutations have the worst outcome (Xing, 2010). Second, transgenic mice models confirmed the weaker carcinogenic potential on the thyroid gland of *RET/PTC* rearrangements (Jhiang et al., 1996; Santoro et al., 1996; Powell et al., 1998) compared to other alterations such as *BRAF* (Knauf et al., 2005) and *RAS* (Vitagliano et al., 2006) mutations. Third, as mentioned above, the low expression levels of the chimeric oncogenes *in vivo*, driven by weak promoters of the *RET* fusion partner genes, hamper their transforming potentials (Richardson et al., 2009).

CONCLUSION

In this review, we have tried to summarize 25 years of research on *RET/PTC* rearrangement started with its discovery in 1987 (Fusco et al., 1987). Many points regarding its properties of dominant oncogene, its epidemiology, the molecular mechanisms of its formation, and its transforming features have been clarified. Doubts remain as to the best laboratory method for its detection, on its real prevalence and on its impact on clinical behavior of PTC. Moreover, more complete information on activated signal transduction pathways, induced gene expression modification and influences on tumor microenvironment still need to be collected. However, what we have learned up to now is sufficient to target *RET/PTC* with potentially specific small molecules that inhibit its tyrosine kinase function (TKIs) in RAI-refractory advanced PTCs or to develop new compounds with these features. In the near future we expect that a deeper knowledge of the key biological events that drive *RET/PTC* induced carcinogenesis will allow us to obtain additional negative modulators of this process to be placed beside *RET/PTC* specific TKIs. In addition, standardization of the detection method and development of multicenter studies might allow clarification of its real prevalence and impact on PTC prognosis.

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Clinical outcome, role of $BRAF^{V600E}$, and molecular pathways in papillary thyroid microcarcinoma: is it an indolent cancer or an early stage of papillary thyroid cancer?

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Most human thyroid cancers are differentiated papillary carcinomas (PTC). Papillary thyroid microcarcinomas (PTMC) are tumors that measure 1 cm or less. This class of small tumors has proven to be a very common clinical entity in endocrine diseases. PTMC may be present in 30–40% of human autopsies and is often identified incidentally in a thyroid removed for benign clinical nodules. Although PTMC usually has an excellent long-term prognosis, it can metastasize to neck lymph nodes; however deaths related to this type of thyroid tumor are very rare. Few data exist on molecular pathways that play a role in PTMC development; however, two molecules have been shown to be associated with aggressive PTMC. S100A4 (calcium-binding protein), which plays a role in angiogenesis, extracellular matrix remodeling, and tumor microenvironment, is over-expressed in metastatic PTMC. In addition, the $BRAF^{V600E}$ mutation, the most common genetic alteration in PTC, is present in many PTMC with extra thyroidal extension and lymph node metastasis. Importantly, recently developed selective [e.g., PLX4720, PLX4032 (Vemurafenib, also called RG7204)] or non-selective (e.g., Sorafenib) inhibitors of $BRAF^{V600E}$ may be an effective treatment for patients with $BRAF^{V600E}$ -expressing PTMCs with aggressive clinical-pathologic features. Here, we summarize the clinical outcome, cancer genetics, and molecular mechanisms of PTMC.

Keywords: papillary thyroid microcarcinoma, $BRAF^{V600E}$ mutation, extracellular matrix, angiogenesis, tumor microenvironment, clinical outcome, genetics, neck lymph node metastasis

INTRODUCTION

The vast majority of human thyroid cancers are differentiated and pathological examination reveals that most of these are papillary thyroid cancers (PTC). The long-term prognosis and recommended treatment for patients with PTC are dependent on the stage of disease. Papillary thyroid microcarcinoma (PTMC) are small thyroid tumors (≤ 1 cm in diameter) that belong to the low-risk well-differentiated PTC, which are probably of little clinical significance, and do not affect patient survival (Arem et al., 1999; Lloyd et al., 2004; Pazaitou-Panayiotou et al., 2007; Shah et al., 2007). It is important to distinguish between PTMC in a clinically recognized malignant thyroid micro nodule and an incidental (asymptomatic) PTMC found after thyroidectomy performed for other indications (e.g., benign thyroid diseases) or during thyroid ultrasound. Incidental PTMC has an outstandingly good prognosis and there is nearly no risk of recurrence or death from PTMC (Sugitani and Fujimoto, 1999; Barbaro et al., 2005; McDougall and Camargo, 2007). However, PTMC may be associated with lymph node metastases at presentation and/or neck loco-regional recurrences during follow-up (Pazaitou-Panayiotou et al., 2007).

Papillary thyroid microcarcinomas may be categorized as: (i) PTMC found at autopsy or incidentally at histology, (ii) PTMC

found incidentally during thyroid or neck ultrasound and diagnosed before surgery by cytology on thyroid fine needle aspiration (FNA) material, and (iii) clinical PTMC (i.e., tumors whose presenting symptoms were loco-regional or distant metastases). PTMCs are found in otherwise normal thyroids or in multinodular goiters (MNGs), and they account for nearly 50% of new cases of PTC (PTC accounts for over 80% of all human thyroid cancers; Leenhardt et al., 2004; Davies and Welch, 2006; Xing, 2009).

Papillary thyroid microcarcinomas are also referred to as small, tiny, or minute carcinomas. Some authors suggest that PTMC found at histology should be called “occult” papillary tumors instead of carcinomas to reflect their benignity.

The use of ultrasound (US) in the assessment of thyroid disease has greatly increased the detection of thyroid micro nodules not detected at clinical examination (Ezzat et al., 1994; Papini et al., 2011). Prospective studies have undertaken systematic evaluation of thyroid nodules incidentally discovered by US and have correlated size and the US and color-Doppler (CD) findings with the prevalence of cancer and its pathologic staging (Leenhardt et al., 1999). Several recent studies have suggested that US-detected PTMC (Figure 1) is a different disease entity from PTMC detected

at autopsy, based on the histological findings and on the existence of some cases with poor prognosis (Sugitani and Fujimoto, 1999; Yamashita et al., 1999; Papini et al., 2002; Chow et al., 2003a; Pellegriti et al., 2004; Lo et al., 2006). The vast majority of non-palpable thyroid nodules identified by US and color-Doppler display a hypoechoic pattern, irregular margins, microcalcifications, and intranodular vascularity indicating risk for malignancy, which can be confirmed by cytological evaluation of FNA material. Due to the non-negligible prevalence of extra-capsular growth and nodal metastasis, US-guided FNA should be performed on all 8–15 mm hypoechoic nodules with irregular margins, intranodular vascular spots, or microcalcifications. Non-palpable thyroid lesions without US risk factors should be followed up after 6–12 months by repeating clinical and US evaluation (Papini et al., 2002).

TREATMENT AND CLINICAL OUTCOME

Treatment of PTMC has been addressed in both European Thyroid Association (ETA; Pacini et al., 2006) and American Thyroid Association (ATA; Cooper et al., 2009) guidelines. When PTMC is diagnosed preoperatively, routine total, or near-total thyroidectomy is the main treatment to eradicate multifocal disease and decrease overall recurrence rate. ATA guidelines for patients with differentiated thyroid cancer state that the benefit of radioiodine treatment appears to be restricted to patients with larger tumors (>1.5 cm) or residual disease after surgery. There is no evidence that there is any benefit of radioiodine treatment in lower risk patients (defined by the following criteria: PTMC with no extension beyond the thyroid capsule, unifocal tumor, no aggressive histologic subtypes, no extra thyroidal extension or angioinvasion, no local or distant metastases, complete resection of macroscopic tumor, and no ^{131}I uptake outside the thyroid bed on the post-therapeutic whole-body scan if ^{131}I was administered; Jonklaas et al., 2006; Cooper et al., 2009). Instead,

the recommendation for radioactive iodine is supported by a study (Sakorafas et al., 2007) that followed 27 of 380 (7.1%) patients diagnosed with incidental PTMC (mean diameter 4.4 mm) following thyroid surgery for benign thyroid disease (20 patients with MNG, six patients with follicular adenoma, and one patient with nodular hyperplasia; Sakorafas et al., 2007). Patients with cytological preoperative diagnosis of thyroid malignancy were excluded from this study. In 11 patients (40.7%), the tumor was multifocal and in about half of them tumor foci were found in both thyroid lobes. In two patients, PTMC infiltrated the thyroid capsule. Total/near-total thyroidectomy was performed in all these patients. All patients with incidental PTMC received suppression therapy and 20 underwent adjuvant radioactive iodine therapy at a dose of 80–100 mCi based mainly on the presence of multifocal PTMC and infiltration of the thyroid capsule. Follow-up (mean 4.56 years, range 1–12 years) was completed in 25 patients; all of these were alive and disease-free. The authors of this study concluded that PTMC is not an uncommon incidental finding after surgery for benign thyroid lesions and that therefore the possibility of PTMC (in particular multifocal incidental PTMC) should be considered in the management of patients with benign thyroid disease. Also, total/near-total thyroidectomy should be considered in patients with apparently benign nodular thyroid disease who exhibit risk factors including findings from history and clinical examination (i.e., development of hoarseness, progressive dysphagia or shortness of breath, rapid growth of the thyroid nodule, especially if observed under thyroid hormone suppressive therapy, or presence of cervical lymphadenopathy) or suspicious findings on preoperative imaging evaluation.

Importantly, whether individual tumor foci in patients with multifocal PTC or PTMC are clonally related or they arise independently is still controversial. While Shattuck et al. (2005) demonstrated that individual tumor foci in patients with multifocal PTC often arise as independent tumors, McCarthy et al. (2006)

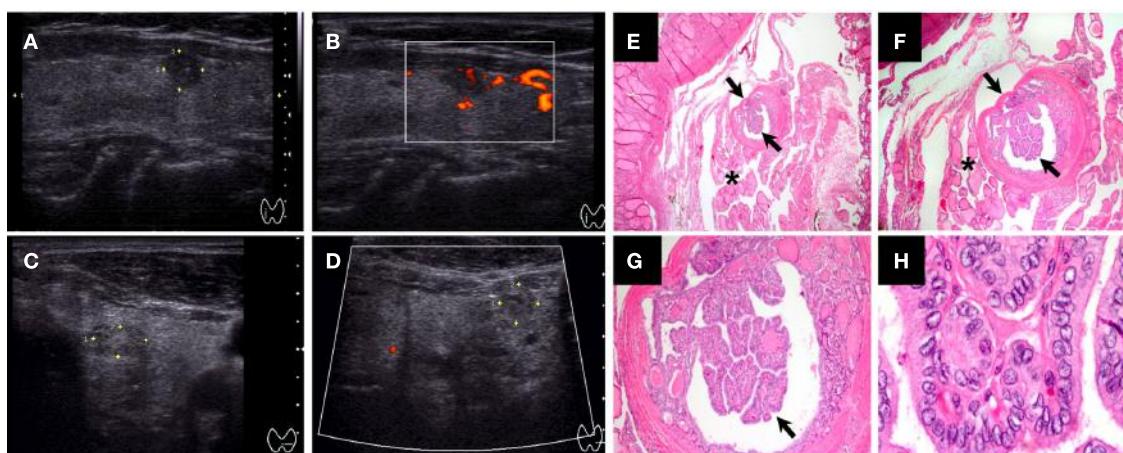


FIGURE 1 | Ultrasound and histological characteristics of a papillary thyroid microcarcinoma (PTMC) nodule. **(A–B)** Papillary thyroid microcarcinoma (PTMC) nodule (5.8 mm) with hypoechoic pattern, microcalcifications, well-defined margins, and intrinsic vascularity. **(C–D)** PTMC nodule (6.3 mm) with isoechoic or mixed echo texture, cystic elements, irregular margins, hypovascularity, and coarse or peripheral

calcifications [also “taller than wider” in **(D)**]. **(E–H)** Histological features of 2 mm encapsulated PTMC with classical-type features in a multinodular goiter [arrows, **(E–G)**]: papillary structure with fibrovascular stalk, nuclear grooves, and nuclear pseudo inclusions **(H)**. The tumor cells had pale eosinophilic cytoplasm with the characteristic vesicular to ground glass appearing nuclei **(H)** [Magnification: **(E)** 20 \times , **(F)** 40 \times , **(G)** 100 \times , and **(F)** 1000 \times].

demonstrated that multifocal PTC often arise from the same clone. Thus, intrathyroid metastasis may play an important role in the spread of PTC and PTMC (McCarthy et al., 2006), but the origin of multifocal tumors is unclear. Remarkably, some studies (Baudin et al., 1998; Chow et al., 2003a) have reported that the number and the size of tumor foci in PTMCs correlated with lymph node metastases at clinical presentation and with recurrence, suggesting that those pathologic features may represent relevant prognostic indicators in patients with PTMC. In fact, PTMC multifocality can be associated with high frequency of bilateral involvement regardless of tumor size (Baudin et al., 1998). Even when there is extended disease, the prognosis of patients with PTMCs is excellent, and the majority of studies report a mortality rate between 0 and 0.4% and a recurrence rate between 1.4 and 7.3% (Pazaitou-Panayiotou et al., 2007). However, there is some controversy regarding clinical outcome and treatment of PTMC, as evidenced in the following selected studies:

- (i) Ito et al. (2003) followed 162 patients with lesions ranging from 3 to 10 mm. In 58 of these, they studied the size of PTMC by ultrasound over 5 or more years. They found no change in 60.3% of these cases, a decrease in 12.1%, and, in two patients, no identifiable cancer in two consecutive ultrasounds. Ito et al. (2004) also reported that PTMCs ≥ 7 mm were more likely to show lateral neck metastasis, suggesting that detailed US examination for lateral metastasis is necessary in patients with a tumor measuring ≥ 7 mm.
- (ii) Hay et al. (1992) evaluated 535 patients with PTMC with a median tumor size of 8 mm. Two patients (0.4%) died, and the 20-year tumor recurrence rate was 6%.
- (iii) Roti et al. (2006) reported on 243 patients diagnosed with PTMC. One group consisted of 52 patients diagnosed with incidental PTMC following thyroidectomy for benign thyroid disease. The second group included 191 patients who underwent thyroidectomy because FNA biopsy of a thyroid nodule and/or clinical evaluation was diagnostic or suspicious for malignancy. No significant differences in clinical and histo-pathological characteristics were observed between the two groups. Mean PTMC diameter was 0.55 ± 0.26 cm in the incidental group and 0.56 ± 0.27 cm in the suspected cancer group. A total of 34 patients had neck node and/or distant metastases at the time of diagnosis. Distant metastases were only significantly observed in patients with PTMC ≥ 8 mm. Thirty-two patients with PTMC with a diameter ≥ 5 mm, and two with PTMC with a diameter < 5 mm had lymph node metastases (Roti et al., 2006). Lymph nodes and distant metastases from thyroid cancers with diameters of 8–15 mm have been also described in other studies (Lin et al., 1997; Sugitani et al., 1998; Nasir et al., 2000). Interestingly, Roti et al. (2006) reported only four patients (1.7%) with recurrent or persistent disease, and no PTMC-related mortality. Similarly, an excellent outcome for PTMC has been described by other investigators (Bramley and Harrison, 1996; Noguchi et al., 1996; Rodriguez et al., 1997; Yamashita et al., 1999; Pelizzo et al., 2004), whereas other studies found persistent or recurrent disease in 6–14.4% of patients with PTMC (Hay et al., 1992; Chow et al., 2003b; Pellegriti et al., 2004).
- (iv) One major issue with PTMC is the threshold value of tumor diameter and whether the outcome for slightly larger thyroid tumors (i.e., 1.1–1.5 cm in diameter) is similar to PTMC. Pellegriti et al. (2004) investigated predictors of relapse in PTMC vs. PTC between 1.1 and 1.5 cm. The authors performed a retrospective study of 299 patients with PTC treated and followed up between 1975 and 2001. Near-total or total thyroidectomy was performed in 292 patients, and lobectomy in seven patients. This study indicated that a high proportion of PTC ≤ 1.5 cm carry one or more risk factors at clinical presentation, including bilateral foci. In particular, authors found that approximately 20% of small (≤ 1.5 cm) PTCs had extra-thyroid invasion and/or bilateral foci. None of the 299 patients in this study died of the disease. However, 43 (14.4%) patients still had persisting/recurrent disease at the last follow-up visit. PTC were subdivided into three groups according to their size (diameter: < 0.5 , 0.6–1.0, and 1.1–1.5 cm) and a progressively increasing frequency of signs of tumor aggressiveness (multifocality, bilaterality, extrathyroidal invasion, and lymph node involvement) which correlated with increasing tumor size. This was particularly evident for PTC greater than 1.0 cm vs. PTC less 1.0 cm in diameter (Pellegriti et al., 2004).
- (v) Presence or absence of risk factors/aggressiveness features are important indicators in planning thyroid surgery for PTMC (e.g., lobectomy alone vs. total thyroidectomy with central compartment neck dissection). Previous studies have shown considerable variability in the prevalence of aggressive features and but significant differences between PTMC and PTC in the prevalence of aggressive features. This was addressed in a recent study (Yun et al., 2010) that found that out of 87 patients (preoperative retrospective study), 44 (51%) had extra thyroidal extension, and 27 (31%) had central lymph node metastasis. Positron emission tomography (PET)/Computed tomography (CT) showed discernible fluorodeoxyglucose (FDG) uptake in 46 PTMCs (53%). FDG positivity of PTMCs was the only variable that correlated with both extra thyroidal extension and central lymph node metastasis; there was a significant difference in the prevalence of both extra thyroidal extension (70 vs. 29%) and central lymph node metastasis (41 vs. 19.5%) between FDG-positive and -negative groups. The authors concluded that visual FDG positivity in PTMCs is a potential risk factor that may be useful for preoperative risk stratification. However, this study lacked long-term follow-up of correlation of FDG positivity in PTMCs with disease relapse rate, thus prospective studies are needed to assess the long-term benefit, cost effectiveness, sensitivity, and specificity of FDG-PET in patients with PTMC.
- (vi) Importantly, Biscola et al. (2004) have reported that PTMC can be associated with medullary thyroid cancer (MTC). Twenty-seven of 196 (13.8%) MTC cases showed an association with PTC, and 21 of 190 (11%) MTC showed an association with incidental PTMC. This percentage is higher than that reported in the literature on the association of PTMC with Graves' disease (GD; 2.8%–4.5%; Schwartz et al., 1989; Mazzaferri, 1990; Hori et al., 1995; Merchant et al.,

2002) or with MNG (3%; Pelizzo et al., 1997). The authors excluded the possibility that this association was caused by an increase in the general frequency of PTMC. Furthermore, although it was not possible to completely exclude a shared pathogenic event as the cause of both MTC and PTMC, the molecular analysis of RET gene alterations did not show any common mutation between these two types of thyroid cancers. The clinical behavior of MTC does not seem to be influenced by the presence or specific therapeutic treatment of a concomitant PTMC (Biscola et al., 2004).

In summary, total or near-total thyroidectomy is the treatment of choice in patients with PTC followed by radioactive iodine ablation, in case of a tumor size greater than 1.0 cm or the presence of lymph node or distant metastasis, to achieve negative ^{131}I whole-body scan and undetectable thyroglobulin (Tg) levels during follow-up. Levothyroxine (LT4) suppressive therapy is recommended for high risk thyroid cancer patients. Post-surgical radioiodine treatment in the case of patients with PTMC (tumor size less or equal to 1 cm in diameter) is clearly indicated in the presence of high risk factors, but can be avoided in patients with low-risk (unifocal tumor, no extra thyroidal extension, no tall cell variant, columnar cell, diffuse sclerosing, and solid/trabecular variants, and no lymph node, or distant metastases; Falvo et al., 2003; Pazaitou-Panayiotou et al., 2007).

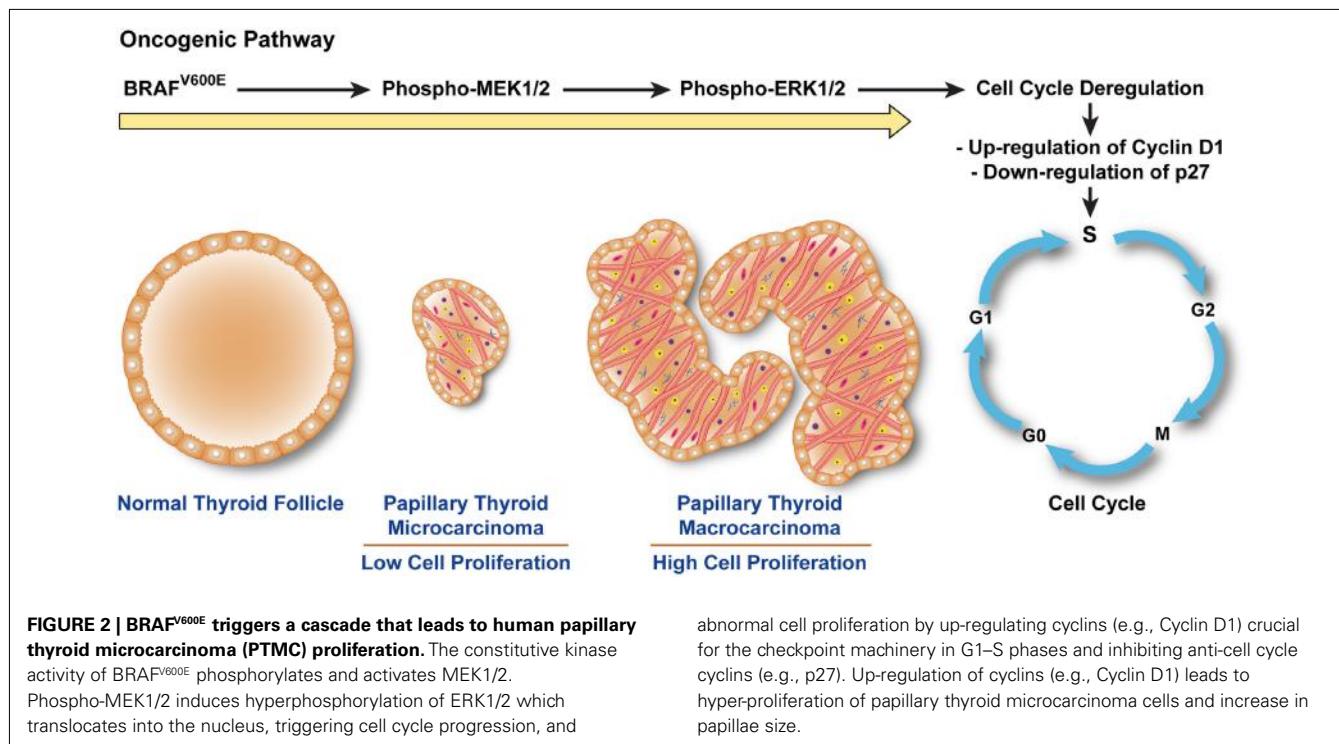
GENETIC ALTERATIONS IN PTMC

The search for molecular targets for PTC has focused primarily on the RET (tyrosine kinase receptor)/RAS/BRAF/MAPK (mitogen-activated extracellular signal regulated kinase, i.e., ERK1/2) kinase

signaling pathway as the oncogenic event in PTC progression (Xing, 2007; Nucera et al., 2010, 2011a,b; Knauf et al., 2011; Ringel, 2011). The major genetic alterations so far described in PTC are translocations in the RET gene (the estimated prevalence is highly variable between different studies, from 3 to 92%, and depends on the experimental methodology used) and the V600E point mutation in the BRAF gene (Nikiforova et al., 2003; Xing, 2007; Marotta et al., 2011), which occurs in about 50% of PTC. Both of these genetic alterations trigger the ERK1/2 pathway, which causes abnormal cell proliferation, adhesion, migration, and invasion (Melillo et al., 2005; Nucera et al., 2010, 2011b; Knauf et al., 2011).

A high prevalence (about 45–52%) of RET/PTC translocations has been reported in PTMCs (Tallini et al., 1998; Corvi et al., 2001), suggesting that the activation of this oncogene (RET/PTC) plays a role in the early stage in PTMC development.

The BRAF^{V600E} mutation appears to occur early in PTC development, based on evidence that it is also harbored in PTMC (Sedliarov et al., 2004; Xing, 2007; Frasca et al., 2008; Nucera et al., 2009) and plays an important role in cell proliferation by regulating cyclins (e.g., cyclins D1 and p27; Motti et al., 2007; Xing, 2007; Salerno et al., 2010; Nucera et al., 2011b; **Figure 2**). Although the overall prevalence of the BRAF^{V600E} mutation in PTC worldwide is relatively high (Xing, 2007), the prevalence of this mutation in PTMC is generally much lower in many parts of the world (Lupi et al., 2007; Ugolini et al., 2007; Frasca et al., 2008) and as low as 18% in PTMC less than 5 mm in diameter (Ugolini et al., 2007). Excluding the areas in Korea where the BRAF^{V600E} mutation in PTMC is high (Xing, 2007), the overall frequency of the BRAF^{V600E} mutation in PTMC is around 30% (Xing, 2007). Lee et al. (2009) studied the clinico-pathological characteristics and



the BRAF^{V600E} mutational status of 64 cases of PTMCs. BRAF^{V600E} mutation was detected in 37.5% of PTMCs. BRAF^{V600E}-positive PTMCs exhibited significantly more features of aggressiveness (advanced disease stages, extra thyroidal extension, and nodal metastasis) than PTMCs without the V600E mutation, indicating that BRAF^{V600E} may be a marker of aggressiveness and tumor progression from PTMC to PTC. Fifty percent of BRAF^{V600E}-positive PTMCs were stage III or IV. These data indicate that the BRAF^{V600E} mutation might be a molecular marker of tumor invasiveness and, moreover, that this relationship is independent of tumor size (e.g., greater than 1.1 cm vs. less than 1 cm).

Recently, Niemeier et al. (2011) analyzed a group of aggressive PTMC selected based on the presence of lymph node metastasis or tumor recurrence and compared with a group of non-aggressive PTMC. The groups were matched for age, sex, and tumor size, but with no extra thyroidal spread (significantly more prevalent in the aggressive group). Importantly, these authors detected BRAF^{V600E} in 77% of aggressive and 32% of non-aggressive PTMCs, suggesting that the V600E mutation may be a marker of invasiveness and, together with histo-pathologic features of aggressiveness, may allow clinical risk stratification of PTMCs.

The BRAF^{V600E} mutation has been correlated with multifocal pathologic features in thyroid cancers (Xing, 2007). As is the case for PTC, the importance of the distinction between multifocal independent primary (IP) PTMC and PTMC with intrathyroid metastasis is unclear. Incidental PTMC can be multifocal (Lin et al., 2008) and associated with lymph node metastases and increased neck lymph nodes recurrence after surgical treatment. Lin et al. (2008) found that the incidence of lymph node metastasis for multifocal PTMC was 42.9%, similar to previous studies (David et al., 1992; Rodriguez et al., 1997; Baudin et al., 1998; Arem et al., 1999). Lin et al. also found that a high percentage (75%) of PTMC with intrathyroid metastasis were positive for lymph node metastasis, while no patients with multifocal IP PTMC had metastatic disease. This suggests that it is important to separate PTMC with intrathyroid metastasis from multifocal IP tumors. In this study, about 50% of multifocal PTMCs showed intrathyroidal metastasis; the molecular profile of this group of PTMCs was characterized by loss of heterozygosities (LOHs) at chromosomes 1p36, 18q21, and 22q13, and/or BRAF^{V600E} mutation, suggesting that both different chromosomal losses and point mutations in the BRAF gene could be important pathological events in the origin of multifocal PTMC.

In summary, there is good evidence that BRAF^{V600E}-positive PTMC are more likely to have a poor prognosis and therefore, it seems reasonable to be more aggressive in treating patients with PTMC carrying the BRAF^{V600E} mutation. Soares and Sobrinho-Simoes (2011) recently suggested that genetic screening for the BRAF^{V600E} mutation might also help assess risk stratification and manage patients with PTMC. PTMC without BRAF^{V600E} may be conservatively managed unless the presence of other markers of poor prognosis indicate a more aggressive therapeutic approach (Xing, 2009).

MOLECULAR PATHWAYS IN PTMC

Since most PTMC progress very slowly while others show more aggressive behavior, it would be clinically relevant to determine a gene signature that can predict tumor aggressiveness. Few studies

have focused on the molecular pathways that underlie the pathobiology of PTMC. Kim et al. (2010) recently performed oligonucleotide array analysis of PTMC and found that cell adhesion molecules were up-regulated in PTMC. In addition, they found no differences in gene expression between PTMC and PTC, suggesting that some PTMC may not be occult indolent thyroid cancers but are an earlier stage of PTC.

Min et al. (2008) found that PTMC with extra thyroidal extension and multifocality exhibited significant expression of S100A4 and that its expression predicted lymph node metastasis. S100A4 (Garrett et al., 2006) is a member of the S100 family of calcium-binding proteins, which includes metastasin, fibroblast-specific protein, pEL-98, 18A2, CAPL, and calvasculin. *In vitro* and *in vivo* studies in rodents have provided evidence that S100A4 is directly involved in tumor progression and metastasis, and promotes angiogenesis. Therefore, preoperative evaluation of the expression of S100A4 in cytological specimens should be helpful in guiding therapy for patients with PTMCs. S100A4 immunoreactivity may predict lymph node metastasis in PTMC and might therefore be useful as an immunohistochemical marker to distinguish between more aggressive PTMC and clinically indolent PTMC.

TARGETED THERAPIES WITH SELECTIVE AND NON-SELECTIVE INHIBITORS OF BRAF^{V600E}

Chemotherapies for metastatic thyroid carcinomas have been of limited effectiveness. Therefore, novel therapies are needed to improve disease outcome for patients with these cancers. Studies based on preclinical models of targeted therapies highlight the importance of individualized genomic profiling to guide patient selection for inclusion in clinical trials.

Some drugs that target the BRAF^{V600E} oncprotein kinase have recently begun clinical trials in patients with melanoma. Selective pharmacologic targeting of BRAF^{V600E} may prove effective for treating patients with PTC harboring this mutation. For example, PLX4720 and PLX4032 are novel orally available selective small molecule inhibitors specifically designed to insert into the ATP-binding site and trap oncogenic BRAF^{V600E} in an inactive conformation (Tsai et al., 2008; Bollag et al., 2010). These compounds inhibit BRAF^{V600E} kinase activity in melanoma, thyroid cancer, and colorectal cancer cells (Tsai et al., 2008; Nucera et al., 2010). PLX4032 (Vemurafenib) induced complete or partial tumor regression in 81% of patients enrolled for phase I–II clinical trial who had melanoma with the BRAF^{V600E} mutation (Flaherty et al., 2010). In addition, it significantly improved rates of overall survival and progression-free survival (74%) in phase III clinical trials in BRAF^{V600E}-positive melanoma patients (Chapman et al., 2011). The effect of PLX4720 in a preclinical model of metastatic human thyroid cancer suggests that these inhibitors might be effective for treating patients with BRAF^{V600E}-positive thyroid cancers that are refractory to conventional therapy (Nucera et al., 2010).

Interestingly, overexpression of angiogenic signaling cascade pathways has been described in human PTC, and preclinical models have shown that inhibition of key molecules (i.e., protein kinases) in these pathways can have anti-tumor effects (Gild et al., 2011). Some of these kinase inhibitors have now been tested in clinical trials, with modest results. For example, Sorafenib was designed as a c-RAF inhibitor; however, it has been reported to

target other kinases, including vascular endothelial growth factor receptors (VEGFR) and BRAF^{V600E}, therefore, it has been classified as non-selective inhibitor of BRAF^{V600E}. Sorafenib has been assessed clinically in patients with BRAF^{V600E}-positive or genetically unknown advanced melanoma and did not show any benefit in those clinical trials (Hauschild et al., 2009; Ott et al., 2010; Caronia et al., 2011). Additionally, in phase I-II clinical trials, only 15% of patients with metastatic PTC showed a partial response to Sorafenib (Kloos et al., 2009; Caronia et al., 2011).

Although only a small percentage of all PTMC are metastatic, ~77% of BRAF^{V600E}-positive PTMC show features associated with aggressiveness (i.e., extra thyroidal extension; Niemeier et al., 2011). For these tumors, targeted therapies based on selective inhibitors of BRAF^{V600E} could be effective in the near future.

CONCLUSION AND PERSPECTIVES

The management and treatment of malignant thyroid micro nodules (i.e., PTMC) can be a challenge for physicians. Most PTMC are indolent and have an excellent prognosis; however, a subgroup shows an aggressive biological and clinical behavior similar to PTC. While additional robust prospective studies are required, there is now a body of evidence suggesting that BRAF^{V600E}-positive PTMCs show aggressive behavior, whereas BRAF^{V600E}-negative PTMCs have a good prognosis. This suggests that it will be valuable to consider the BRAF^{V600E} mutation as a prognostic marker of PTMC aggressiveness and to undertake prospective studies with systematic screening for the BRAF^{V600E} mutation and long-term follow-up to validate this marker of tumor aggressiveness.

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A validated marker would help endocrinologists and oncologists stratify clinical risk in PTMC. Although, additional biological studies are needed to address definitively whether PTMC is an indolent cancer or an early stage of PTC, current clinical and molecular data suggest that patients with PTMCs that harbor the BRAF^{V600E} mutation and exhibit aggressive clinical-pathologic features may benefit from treatment with BRAF^{V600E}-selective inhibitors. There is sufficient promising preclinical experimental data to suggest that physicians could begin to test such targeted treatments now, preferably in the context of a prospective clinical trial following patients with aggressive BRAF^{V600E}-expressing PTMC.

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Descriptive epidemiology of human thyroid cancer: experience from a regional registry and the “volcanic factor”

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Thyroid cancer (TC), the most common endocrine tumor, has steadily increased worldwide due to the increase of the papillary histotype. The reasons for this spread have not been established. In addition to more sensitive thyroid nodule screening, the effect of environmental factors cannot be excluded. Because high incidences of TC were found in volcanic areas (Hawaii and Iceland), a volcanic environment may play a role in the pathogenesis of TC. In January 2002, the Regional Register for TC was instituted in Sicily. With a population of approximately five million inhabitants with similar genetic and lifestyle features, the coexistence in Sicily of rural, urban, industrial, moderate-to-low iodine intake, and volcanic areas provides a conducive setting for assessing the environmental influences on the etiology of TC. In Sicily, between 2002 and 2004, 1,950 new cases of TC were identified, with an age-standardized rate (world) ASR(w) = 17.8/10⁵ in females and 3.7/10⁵ in males and a high female/male ratio (4.3:1.0). The incidence of TC was heterogeneous within Sicily. There were 2.3 times more cases in the Catania province (where most of the inhabitants live in the volcanic area of Mt. Etna): ASR(w) = 31.7/10⁵ in females and 6.4/10⁵ in males vs. 14.1 in females and 3.0 in males in the rest of Sicily. Multivariate analysis documented that residents in the volcanic area of Mt. Etna had a higher risk of TC, compared to the residents in urban, industrial, and iodine deficient areas of Sicily. An abnormally high concentration of several chemicals was found in the drinking water of the Mt. Etna aquifer, which provides water to most of the residents in the Catania province. Our data suggest that environmental carcinogen(s) of volcanic origin may promote papillary TC. Additional analyses, including cancer biological and molecular features, will allow a better understanding of risk factors and etiopathogenetic mechanisms.

Keywords: thyroid cancer, epidemiology, registry, incidence, risk factors, papillary, volcanoes

INTRODUCTION: THYROID CANCER EPIDEMIOLOGY AND TREND

Thyroid cancer (TC) is the most frequent endocrine neoplasm (Curado et al., 2007), which occurs two to four times more frequently in females than in males (Parkin et al., 2005). Its incidence has continuously increased worldwide in recent decades. This increase has occurred in many regions, with variable entity and independent of initial recorded levels, as indicated by data from population-based cancer registries, particularly in the Western world, such as the United States (Davies and Welch, 2006), Canada, and in many European Countries (Leenhardt et al., 2004; Yu et al., 2010) (Table 1).

Based on data from 1999 through 2008, TC represents the fastest growing cancer, in terms of prevalence, in the United States among men and women of every racial and ethnic background (except American Indian or Alaska Native men) (Simard et al., 2012).

The epidemiological data of Surveillance Epidemiology and End Results (SEER) reported an annual incidence of TC of 5.9/10⁵ individuals in men and 17.3/10⁵ in women from 2005 through 2009 and an age-adjusted death rate of 0.5/10⁵ in both men and women per year (Howlader et al., 2012).

The reasons for the continuously increasing incidence of TC are unclear and controversial. The increase in TC might be attributed to more intensive and sensitive thyroid nodule diagnostic procedures (thyroid ultrasound and fine-needle aspiration biopsies) and the results of improved diagnosis of subclinical cancers (Davies and Welch, 2006; Kent et al., 2007). Methodologies for the pathological examination of the excised thyroid gland could also explain this phenomenon. Recently, pathologists have begun to dissect thinner slices of the gland (less than 2 mm) and frequently sample not only macroscopically evident lesions but also the entire thyroid gland (Pazaitou-Panayiotou et al., 2007; Boucek et al., 2009).

Table 1 | Age-adjusted thyroid cancer incidence rates from different population-based cancer registries.

Authors	Data source	Study period	No. of cases		Incidence rates*	
			Males	Females	Males	Females
Chen et al. (2009)	SEER	1988–2005	7,458	23,308	2.5–5.1	6.4–14.9
Burgess (2002)	ANCSCH	1982–1997	–	–	1.27–2.04	2.89–5.52
Scheiden et al. (2006)	MTR	1983–1999	74	236	3.0–5.7	9.0–10.5
Sassolas et al. (2009)	TCR-RA	1998–2006	1,256	4,111	2.47–3.95	8.35–12.70
Rego-Iraeta et al. (2009)	PRUHV	1978–2011	70	252	0.33–3.24	1.56–8.23
Netea-Maier et al. (2008)	NCR	1989–2003	1,451	3,629	0.9–1.1	3.1–3.1
AIRTUM Working Group (2006)	AIRTUM	1998–2002	675	2,579	5.2	15.5
Pellegriti et al. (2009)	SRRTC	2002–2004	366	1,584	3.7	17.8

*Range provides incidence at the beginning and end of the study period.

SEER, Surveillance Epidemiology and End Results; ANCSCH, Australian National Cancer Statistics Clearing House; MTR, Morphologic Tumor Registry (Luxembourg); TCR-RA, Thyroid Cancer Registry of Rhône-Alpes region (France); PRUHV, Pathology Registry of the University Hospital of Vigo (Spain); NCR, Netherlands Cancer Registry; AIRTUM, Italian Association of Cancer Registry; SRRTC, Sicilian Regional Registry for Thyroid Cancer (Italy).

The increased microcarcinoma prevalence and the rising number of incidentally discovered TCs are consistent with these hypotheses (Leenhardt et al., 2004; Davies and Welch, 2006). Recently, it has been hypothesized that environmental factors (or chemical agents) might explain this increasing incidence (Leenhardt and Grosclaude, 2011) and, consequently, that a more accurate screening cannot explain the (likely) multifactorial mechanisms that might account for this increase.

To the best of our knowledge, there are no available data in the recent literature, but we can hypothesize that the more diffuse use of sensitive diagnostic procedures has reached a plateau in many countries because of standardized diagnostic protocols. The incidence of TC has not been steady, as reported in the case of prostate cancer, according to data from the SEER registries (annual percentage change of -1.5 in the period 2000–2009) (Howlader et al., 2012).

The relevant increase of TC generally refers to papillary cancer less than 1 cm in diameter (microcarcinoma), which leads to an “epidemic of micropapillary TC” (How and Tabah, 2007). A recent article emphasized that the most common type of TC in patients older than 45 years is microcarcinoma (Hughes et al., 2011). Nevertheless, the incidence of TCs of all sizes (including the large ones, ≥ 4 cm in diameter) increased in the 1988–2005 period in the United States (Chen et al., 2009) as well as in other countries (Rego-Iraeta et al., 2009).

Furthermore, the increased incidence of TC is nearly exclusively due to the papillary histotype, with no significant change in the other histotypes. This finding suggests that specific carcinogens might favor the molecular abnormalities typical of papillary cancer.

Controversy has emerged regarding the differences in the incidence of TC in different ethnic groups. In addition to the possible effect of genetic factors, variable access to medical care has been hypothesized to be a contributing to this incidence. Although the incidence of TC is lower among Hispanics and Afro-Americans compared to Caucasians (Simard et al., 2012), very recent data report the greatest acceleration in incidence rate of papillary TC among black females (Aschebrook-Kilfoy et al., 2013).

At present, carcinogens that may trigger TC or contribute to its progression have not been established. The risk factors for TC include exposure to ionizing radiations (Nikiforov, 2006), a history of goiter or thyroid nodules or a family history of TC. In addition, dietary factors (Markaki et al., 2003), westernized lifestyle and obesity (Kitahara et al., 2011), or anthropogenic pollution (i.e., caused by human activities) cannot be excluded as factors. Concerning dietary factors, several studies have investigated the impact of iodine intake on the risk of TC onset. Whether adequate iodine consumption has a protective role against thyroid carcinogenesis has not been determined. These studies concur with the observation that the ratio of papillary to follicular TCs is higher in geographical areas with adequate iodine intake than in areas with moderate-to-severe iodine deficiency (Peterson et al., 2012).

Some of the highest incidences of TC were found in Hawaii (Goodman et al., 1988; Kolonel et al., 1990) and Iceland (Arnbjornsson et al., 1986), both volcanic areas. A comparison of the same ethnic groups living in Hawaii or in other geographical areas indicated that the incidence of TC was significantly higher in the first group, which suggests that environmental factors might play a role (Goodman et al., 1988).

Large population-based cancer registries are appropriate for studying the incidence of cancer and genetic factors in relation to the environment. These registries require a close cooperation between clinicians, epidemiologists, and environment specialists.

Creating such a registry was the aim of our research; the study began planning early in 2000 on the Mediterranean island of Sicily.

THE SICILIAN REGIONAL REGISTRY FOR TC

Sicily, with approximately five million inhabitants, offers the opportunity to study different environmental factors that can influence the incidence of TC. Sicily has a homogeneous population in terms of genetics and lifestyle, with similar access to medical assistance and, as an island, is very well geographically delimited. In Sicily, there is the concomitant presence of rural and urban areas, industrial and non-industrial areas, and areas of low or adequate iodine intake. Sicily also hosts Mt. Etna, the highest and most active volcano in Europe.

Data collection was organized through the institution of the Sicilian Regional Registry for Thyroid Cancer (SRRTC). Patients were identified by a systemic survey (three to four times each year) of all Sicilian (public and private) morbid pathology services. Data were recorded in a computerized archive and were correlated with population data (Italian National Institute of Statistics, 2001) (Pellegriti et al., 2009).

We evaluated the age-standardized incidence rate for the world population (ASR_w) in each of the nine provinces in Sicily in the 3-year period of 2002–2004. We found a more than twofold increased incidence of TC in the province of Catania ($ASR_w F = 31.7/10^5$; $M = 6.4/10^5$), where approximately 80% of the residents live in the volcanic area of Mt. Etna, in contrast to the rest of Sicily ($ASR_w F = 14.1/10^5$; $M = 3.0/10^5$) (Table 2). The incidence of TC was not different between the industrial and the non-industrial areas nor between the areas of sufficient and deficient iodine intake. A higher ASR_w was observed in urban areas compared to rural areas. A similar finding was reported by Sassolas et al. (2009), who documented in France a higher incidence of TC in the urban cantons of the Rhône-Alpes region than in the non-urban areas of the same region. A non-uniform presence of medical specialists between the urban and non-urban areas may explain this difference. Although a different access to medical assistance between the urban and rural areas is reasonable, this variable access is not likely to explain the difference between the Catania province and the other eight Sicilian provinces. The Catania province includes the Catania metropolitan area (approximately 650,000 inhabitants) and a large rural area with approximately 350,000 inhabitants. A similar urban/rural ratio is present for the provinces of Palermo and Messina, where the incidence of TC is much lower than in Catania. In Sicily, a similar access to medical assistance is indicated by the number of routine biochemical laboratory tests per inhabitant: 6.89, 7.42, and 6.18 in 2007 in the provinces of Catania, Messina, and Palermo, respectively (Sicilian Public Health Report 2008, Appendix 1, www.gurs.regione.sicilia.it). These three

provinces include the largest Sicilian cities, approximately 60% of the Sicilian population, as well as the largest medical schools and major hospitals. Moreover, the Poisson regression analysis adjusted for all environmental characteristic confirmed that, compared with residents of urban, industrial, and iodine deficient areas, living in the volcanic area of the province of Catania was an independent risk factor of TC in both females (odds ratio = 2.242, 95% C.I. = 2.020–2.488) and males (odds ratio = 2.052, 95% C.I. = 1.652–2.565) inhabitants (Table 3).

The mean age of the patients at the time of diagnosis and the female/male ratio were similar between the Catania province and the other Sicilian provinces. Most of TCs in the Catania province were papillary (94%), whereas in Sicily, excluding the Catania province, the percentage of papillary TC was lower (86%) ($p < 0.0001$). Although the mean tumor size at the time of diagnosis was slightly but significantly higher in Sicily, excluding Catania, with respect to the Catania province (1.50 ± 0.04 vs. 1.30 ± 0.05 cm; $p = 0.002$), the occurrence of both extrathyroid extension and lymph node metastases and the stage classification of tumors were not different between the two areas. Curiously, a higher percentage of multifocal TC was observed in the Catania province; i.e., 32 vs. 25% in the rest of Sicily ($p = 0.001$).

VOLCANIC ACTIVITY: A POSSIBLE RISK FACTOR FOR TC

The presence of a high incidence of TC in volcanic areas in different parts of the world suggests that factors are present in the volcanic environment that may act as endocrine disruptors and carcinogens.

Several studies analyzed the incidence of TC among people living in the volcanic areas of Iceland (Hrafnkelsson et al., 1989), Hawaii (Kolonel et al., 1990), New Caledonia (Truong et al., 2007), and French Polynesia (Curado et al., 2007) and documented some of the highest occurrence rates reported in the literature. In French Polynesians, for example, the ASR_w are $5.4/10^5$ for males and $37.4/10^5$ for females. How the volcanic environment may influence

Table 2 | Thyroid cancer incidence in Sicily: age-standardized rates for the world population (ASR_w) for the entire island and by environmental characteristic.

Environmental characteristic	No. of residents	Females			Males			p^*
		no. of cases	ASR_w	(95% C.I.)	no. of cases	ASR_w	(95% C.I.)	
Total for all Sicily	4,980,352	1584	17.8	16.9–18.7	366	3.7	3.3–4.1	
Volcanic environment								
Yes (Catania Province)	1,059,811	599	31.7	29.1–34.3	130	6.4	5.2–7.5	<0.001
No (Sicily without Catania Province)	3,920,541	985	14.1	13.2–15.0	236	3.0	2.6–3.4	
Rural	1,133,529	332	16.4	14.6–18.2	73	3.3	2.5–4.3	0.003
Urban	3,846,823	1252	18.2	17.2–19.2	293	3.9	3.4–4.3	
Industrial	364,110	93	14.1	11.2–17.0	26	3.8	2.2–5.4	0.005 [†]
Non-industrial	4,616,242	1491	18.1	17.2–19.0	340	3.7	3.3–4.1	
Iodine deficiency	208,512	68	19.2	14.4–23.8	18	4.4	2.2–6.6	0.0084
Iodine sufficiency	4,771,840	1516	17.7	16.8–18.4	348	3.7	3.3–4.1	

^{*}Poisson regression adjusted for sex and age.

[†]When excluding volcanic area (where no industrial zone is present) $p = 0.46$.

Table 3 | Thyroid cancer incidence in Sicily: odds ratio by environmental characteristic.

Environmental characteristic	No. of residents	Females			Males			p*
		no. of cases	OR	(95% C.I.)	no. of cases	OR	(95% C.I.)	
Volcanic environment								
Yes (Catania Province)	1,059,811	599	2.242	2.020–2.488	130	2.052	1.652–2.565	<0.001
No (Sicily without Catania Province)	3,920,541	985	1		236	1		
Urban	3,846,823	1252	1.056	0.929–1.200	293	1.118	0.852–1.465	N.S.
Rural	1,133,529	332	1		73	1		
Industrial	364,110	93	0.998	0.805–1.237	26	1.174	0.773–1.784	N.S.
Non-Industrial	4,616,242	1491	1		340	1		
Iodine deficiency	208,512	68	1.163	0.900–1.502	18	1.468	0.897–2.401	N.S.
Iodine sufficiency	4,771,840	1516	1		348	1		

*Poisson regression adjusted for all environmental characteristic.

thyroid carcinogenesis is not known, but non-anthropogenic carcinogens of volcanic origin may be responsible (Kung et al., 1981; Duntas and Doumas, 2009). The geologic processes of volcanism produce various elements and metals in abnormal concentrations in the soil, water, and atmosphere. Mt. Etna continuously delivers, and has for many decades, suspended particulate matter and gases: sulfur dioxide, hydrogen chloride, hydrogen fluoride, hydrogen sulfide, hydrochloric acid, sulfuric acid, ammonium sulfate, helium, and radon. These substances were detected in various volcanic eruptions (Hansell and Oppenheimer, 2004). Many of these potentially toxic compounds may become concentrated the environment (Kusky, 2008) and contaminate cultivated fields, including cultured vegetables and the animal food chain (Hogan and Burstein, 2007).

Several studies reported that various elements and chemicals (including HCO_3 , SO_4 , calcium, selenium, fluoride, chloride, magnesium, boron, manganese, iron, and vanadium and their salts and 222 radon) are often increased in water samples from various sources of Mt. Etna's volcanic aquifer (Giannanco et al., 1996; Brusca et al., 2001; Aiuppa et al., 2002; D'Alessandro and Vita, 2003). Similar findings were reported in studies of water samples from other volcanic areas (Tilling and Jones, 1996; Martin-Del Pozzo et al., 2002). Mt. Etna is a large basaltic volcano with fissured and highly permeable lava layers interbedded with discontinuation layers of scarcely permeable pyroclastics. The main aquifers of Mt. Etna (overall calculated as 1.7 million cubic meters of water) lie between the volcanic rocks and the underlying impermeable sediments. A magmatic-type interaction occurs between the water and the volcanic soil; an enormous amount of CO_2 produced by the volcanic degasification leads to the acidification of water and chemicals leaching from the basalt rock, especially in the lower south-southwestern and eastern flanks of the volcano (Brusca et al., 2001). Vanadium, for instance, is classified by the International Agency for Research on Cancer (IARC) as a possible human carcinogen (Group 2B) (IARC, 2006), which may influence thyroid function and cell proliferation. Experiments on rats documented the role of vanadium in affecting iodine metabolism and thyroid function by decreasing the thyroid peroxidase activity

(Uthus and Nielsen, 1990). Vanadium may have a mitogen effect by stimulating the action of an unknown growth factor (Zhang et al., 2001; Ingram et al., 2003). Approximately 700,000 residents in the province of Catania receive water from the volcanic aquifer, and large agricultural areas are irrigated with water originating from the Mt. Etna aquifer. The role of water can explain why the increased cancer incidence is not related to the distance from the top of Mt. Etna. Messina ($\text{ASR}_w F = 16.2/10^5$; $M = 4.2/10^5$) and Enna ($\text{ASR}_w F = 15.9/10^5$; $M = 2.8/10^5$), which are adjacent to the Catania province, despite their proximity to Mt. Etna, do not have an increased incidence of TC. Messina and Enna provinces, however, receive most of their water from different aquifers (Peloritani Mountains for Messina and Erei Mountains for Enna) (Pellegriti et al., 2009).

However, the role of the different aquifer systems of the eastern Sicilian provinces has not been confirmed. The role of volcanic water as the vehicle for carcinogenic trace elements in initiating and promoting TC requires additional research.

The population's exposure to one or more carcinogens of volcanic origin can occur in other ways, such as atmosphere, soil, or contaminated foods. The preliminary data indicate that the resident population is exposed to an abnormal concentration of trace elements that are increased in biological fluids. Mt. Etna (and other volcanoes) is a large emitter of trace elements; metals, separated from magma during degassing, are transported by rising gases, and as they approach the surface, they condense to small particles that are dispersed throughout the atmosphere. Because of the constant degassing and recurrent lava flow eruptions, atmospheric emissions of particulate Cd, Hg, Se, Cu, and Zn by Mt. Etna are equivalent to the amount of these elements released in the Mediterranean area by all of the anthropogenic activities (Buat-Ménard and Arnold, 1978). Most common winds in the Mt. Etna region blow from the north-northwest (Favalli et al., 2004). The Mt. Etna plume, therefore, moves mainly south-east, and Catania province inhabitants mostly live in the downwind areas of the ash fallout. Therefore, these individuals are exposed to high levels of these particulates. Moreover, vegetables and plants may accumulate various trace elements dispersed in the atmosphere. Several studies

Table 4 | Summary of the principal studies quoted in the manuscript.

Topic of the study	Authors	Years of the study	Geographical area	Main conclusions
Trace element concentration in water of volcanic aquifers	Tilling and Jones (1996)	1973–1991	Mt. Kilauea (Hawaii)	The overall chemistry of the volcanic aquifer is largely due to a hydrolysis reactions leading to the leaching of the rocks and is dependent on the volcanic degassing of CO_2
	Martin-Del Pozzo et al. (2002)	1994–2000	Mt. Popocatepetl (Mexico)	Spring water content of SO_4^{2-} , Cl^- , F^- , HCO_3^- , B , SO_4^{2-} / Cl^- , Na^+ , Ca^{2+} , SiO_2 , and Mg^{2+} measured before and during eruptions, show response to volcanic activity
	Giammanco et al. (1996)	1994–1997	Mt. Etna (Sicily)	Concentration of trace elements in groundwaters of Mt. Etna is temporally variable, linked to the volcanic activity
	Aiuppa et al. (2002)	1997–1999	Mt. Etna (Sicily)	Concentrations of B , V , and Mg in groundwaters of Mt. Etna exceed the maximum admissible limits
Trace element concentration in vegetables grown in volcanic areas	Barghiani et al. (1987)		Mt. Etna (Sicily)	Hg content is high in <i>Pinus</i> of the Mt. Etna area
	Queirolo et al. (2000)		Northern Chile	Concentration of As, Pb, and Cd are high in locally cultivated vegetables
	Abiye et al. (2011)		Ethiopia	Geogenic sources of Cd, Cr, Pb, and Zn are responsible for the high concentration of these metals in locally grown vegetables
	Dahal et al. (2008)		Nepal	The arsenic content in soil and plants is influenced by the degree of arsenic amount in irrigation water
Thyroid cancer incidence in volcanic areas	Goodman et al. (1988)	1960–1984	Hawaii	$M = 3.1/10^5$; $F = 8.1/10^5$
	Hrafnkelsson et al. (1989)	1955–1984	Iceland	$M = 3.4/10^5$; $F = 9.5/10^5$
	Truong et al. (2007)	1985–1999	New Caledonia	$M = 10.4/10^5$; $F = 71.4/10^5$
	Curado et al. (2007)	1998–2002	French Polynesia	$M = 5.4/10^5$; $F = 37.4/10^5$
	Pellegriti et al. (2009)	2002–2004	Mt. Etna (Sicily)	$M = 6.4/10^5$; $F = 31.7/10^5$
	Biondi et al. (2012)	2000–2009	Mt. Vesuvius (Campania)	PTC incidence crude rate = $9.0/10^5$ vs. $6.2/10^5$, respectively, in volcanic and non-volcanic area

documented an increased amount of heavy metals in plants grown in volcanic areas. For instance, a significantly enhanced Hg content was found in *Pinus* of the Mt. Etna area (Barghiani et al., 1987). Other researchers found high levels of different trace elements (As, Sb, Bi, Cd, Cr, Pb, Zn) in vegetables grown in areas characterized by volcanic activity (Queirolo et al., 2000; Jung et al., 2002; Abiye et al., 2011). Vegetable contamination may occur not only through the atmospheric pollution but also by the presence of heavy metals in the irrigation water (Dahal et al., 2008).

Finally, environmental carcinogens of volcanic origin could be responsible for gene mutations favoring the thyroid carcinogenesis. In a recent paper, our group reported a higher rate of $\text{BRAF}^{(\text{V600E})}$ in eastern Sicily (hosting the Mt. Etna), compared to western Sicily (Frasca et al., 2008).

Table 4 provides a summary of the principal studies quoted in the present manuscript.

CONCLUSION

The rising number and the diffusion of sensitive diagnostic procedures may justify only part of the continuous increase of TC incidence. Additional studies are necessary to investigate the role of environmental factors, which may include the volcanic environment. Population-based cancer registries may allow a better understanding of the different agents and mechanisms underlying this increase.

There is a strong association between very high TC incidence and patient residence in volcanic areas. We propose the following considerations: (1) TC registries should be instituted in all volcanic areas with elevated population density because those populations are most likely at high risk for developing TC. However, not all volcanoes are necessarily the same in terms of environmental factors and vehicles for human exposure to carcinogens. (2) A primary aim of the research in this field should be to identify environmental factors and thyroid carcinogens in volcanic areas. These studies will enable us to understand the mechanisms and molecular alterations that might lead to TC. We can then develop specific interventions to prevent the increased risk of TC. (3) It is important to ascertain whether, in addition to TC, other cancers are favored by the volcanic environment. In the Mt. Etna area, an increased incidence of mesothelioma has been reported in the Biancavilla municipality district (Comba et al., 2003). Mesothelioma has been attributed to the increased inhalation of asbestos fibers, a consequence of the generalized use of building materials from a local quarry. In Africa, endemic Kaposi's sarcoma has been observed in areas containing volcanic clay minerals (Ziegler, 1993).

More than 500 million people in the world live in volcanic areas. Identifying the potential carcinogens involved in the pathogenesis of TC might help to promote preventive measures and understand the worldwide increase of this disease.

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Familial follicular cell-derived thyroid carcinoma

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Follicular cell-derived well-differentiated thyroid cancer, papillary (PTC) and follicular thyroid carcinomas comprise 95% of all thyroid malignancies. Familial follicular cell-derived well-differentiated thyroid cancers contribute 5% of cases. Such familial follicular cell-derived carcinomas or non-medullary thyroid carcinomas (NMTC) are divided into two clinical-pathological groups. The syndromic-associated group is composed of predominantly non-thyroidal tumors and includes Pendred syndrome, Werner syndrome, Carney complex (CNC) type 1, *PTEN*-hamartoma tumor syndrome (PHTS; Cowden disease), and familial adenomatous polyposis (FAP)/Gardner syndrome. Other conditions with less established links to the development of follicular cell-derived tumors include ataxia-telangiectasia syndrome, McCune Albright syndrome, and Peutz-Jeghers syndrome. The final group encompasses syndromes typified by NMTC, as well as pure familial (f) PTC with or without oxyphilia, fPTC with multinodular goiter, and fPTC with papillary renal cell carcinoma. This heterogeneous group of diseases does not have the established genotype-phenotype correlations known as in the familial C-cell-derived tumors or medullary thyroid carcinomas (MTC). Clinicians should have the knowledge to identify the likelihood of a patient presenting with thyroid cancer having an additional underlying familial syndrome stemming from characteristics by examining morphological findings that would alert pathologists to recommend that patients undergo molecular genetic evaluation. This review discusses the clinical and pathological findings of patients with familial PTC, such as FAP, CNC, Werner syndrome, and Pendred syndrome, and the heterogeneous group of familial PTC.

Keywords: familial thyroid carcinoma, familial adenomatous polyposis, Cowden syndrome, Carney complex, Pendred syndrome, Werner syndrome, familial papillary thyroid carcinoma

INTRODUCTION

Malignant thyroid tumors account for 1% of all malignant tumors (American Cancer Society, 2007). The incidence of thyroid malignancy has increased more than that of any other cancer (Horner et al., 2009). Newly reported cases of thyroid cancer in the United States grew from 18,000 in 2000 to 35,000 in 2007 (Horner et al., 2009). This is likely due to advancements in diagnostic techniques and early detection of small thyroid nodules (microcarcinomas), which clinical meaning is uncertain (Zhu et al., 2009). Almost 95% of patients have well-differentiated cancer of follicular cell origin, including papillary (80–90%), and follicular (10–15%). Approximately 5% of patients have medullary thyroid cancer.

Advances in molecular genetics have also confirmed the presence of several familial cancer syndromes with non-medullary familial thyroid cancers, usually papillary or follicular cancers. These include familial adenomatous polyposis (FAP), Cowden syndrome (CS), Werner syndrome, Carney complex (CNC), and Pendred syndrome (Dotto and Nósé, 2008; Nósé, 2008; Vriens et al., 2009).

Familial follicular cell-derived tumors may account 5–15% of thyroid carcinoma cases. A classification of familial follicular cell thyroid cancer associated with hereditary syndromes (Dotto and Nósé, 2008; Nósé, 2008) is shown in Table 1. The inheritance, gene involved, and risks of familial cancer syndromes for developing thyroid cancer are also shown in Table 1.

FAMILIAL ADENOMATOUS POLYPOSIS

Familial adenomatous polyposis is inherited as an autosomal dominant trait caused by a germline mutation in the adenomatous polyposis coli (APC) gene. The APC gene is a tumor suppressor gene on chromosome 5q21 (Spigelman et al., 1989; Luk, 1995; Cetta et al., 2000). Patients typically have thousands of adenomas that are primarily located in the colon and rectum. Virtually all patients will progress to colorectal cancer if FAP is not identified and treated surgically with proctocolectomy. Most patients also develop gastric polyps of the fundus, most of which do not progress to carcinoma. Polyps in the duodenum and periampullary region are adenomatous, with an increased risk for progression to cancer estimated to be 200 times greater than that of patients in the general population (Spigelman et al., 1989; Luk, 1995; Cetta et al., 2000).

Patients may have extraintestinal manifestations that include osteomas, dental abnormalities, epidermal cysts, desmoids tumors, congenital hypertrophy of the retinal pigment epithelium (CHRPE), hepatoblastoma, medulloblastoma, and thyroid cancers.

Patients with FAP are at risk for developing papillary thyroid carcinoma (PTC). PTC is one of the extracolonic manifestations of FAP. Young women with FAP are at particular risk of developing thyroid cancer, with risk approximately 160 times higher than that of normal individuals, and PTC occurs with a frequency about

Table 1 | Hereditary tumor syndromes associated with thyroid cancer.

Disease	Histological type	Gene mutation	Location	Incidence of thyroid cancer	Pathological variant of PTC
FAP and Gardner syndrome	PTC	APC tumor suppressor gene	5q21	2–12%	Cribiform-morular classical variant
Cowden syndrome	FTC, PTC, C-cell hyperplasia	PTEN tumor suppressor gene	10q23.2	>10%	
Carney complex	FTC, PTC	PRKAR1-x	2p16 17q22–24	60 and 4%	
Werner syndrome	FTC, PTC, ATC	WRN gene	8p11–p12	18%	

PTC, papillary thyroid cancer; FAP, familial adenomatous polyposis; APC, adenomatous polyposis coli; FTC, follicular thyroid cancer; PTEN, phosphatase and tensin; PRKAR1-x, protein kinase A regulatory subunit type 1-alpha; ATC, anaplastic thyroid cancer; WRN, Werner.

10 times that expected for sporadic PTC (Harach et al., 1994; Cameselle-Teijeiro and Chan, 1999; Soravia et al., 1999). Prevalence ranges from 2 to 12% of patients with FAP (Herraiz et al., 2007).

Thyroid carcinomas associated with FAP are typically bilateral and multifocal, with histological features different from sporadic tumors, with characteristic histopathological cribiform pattern with solid areas and a spindle cell component, and are most often associated with marked fibrosis (Figure 1). The characteristic cellular and nuclear findings of sporadic PTC as grooved, overlapping, and clear nuclei are absent in this subtype (Harach et al., 1994; Cameselle-Teijeiro and Chan, 1999; Soravia et al., 1999).

The cribiform-morular variant of PTC (CMv-PTC) is a very rare subtype of PTC representing 0.1–0.2% of cases, or less than 1 in 500 cases of all papillary carcinoma cases (Harach et al., 1994; Cameselle-Teijeiro and Chan, 1999). The overall prognosis for CMv-PTC is similar to that of classical variants of PTC with less than 10% of cases demonstrating aggressive clinical behavior. Among patients with FAP who have synchronous PTC, over 90% of these cases have been reported to cribiform-morular variant. While not all CMv-PTC is associated with FAP, a significant proportion of cases are associated with FAP. Patients with cribiform-morular variant PTC should be evaluated for FAP. This form of PTC is typically bilateral, presents at a younger age, and is 10 times more common in female patients with FAP. The histology of CMv-PTC is characterized by cribiform, solid, and morular areas lacking typical nuclear features of PTC and CMv-PTC is associated with germline and somatic mutations in the APC and β -catenin genes. In contrast to conventional PTC, CMv-PTC rarely metastasizes and carries a benign prognosis. CMv-PTC is revealed by aberrant nuclear and cytoplasmic expression of β -catenin (β -catenin immunostaining is strong in cytoplasm and nuclei in the morular and cribiform areas, and it is only expressed in cell membrane of the non-tumoral follicular cells; Figure 2).

As with other familial non-medullary thyroid carcinoma (FNMTc) syndromes, the low incidence in FAP patients suggests that PTC occurs primarily as a result of a susceptibility gene. Somatic mutations of RET-PTC1 and RET-PTC3 have been identified. Investigators have also identified differences in the location of APC germline mutations in FAP patients with and without PTC (Cetta et al., 2000). They found that 13/15 (87%) patients with FAP-associated PTC had germline mutations and that 12 of

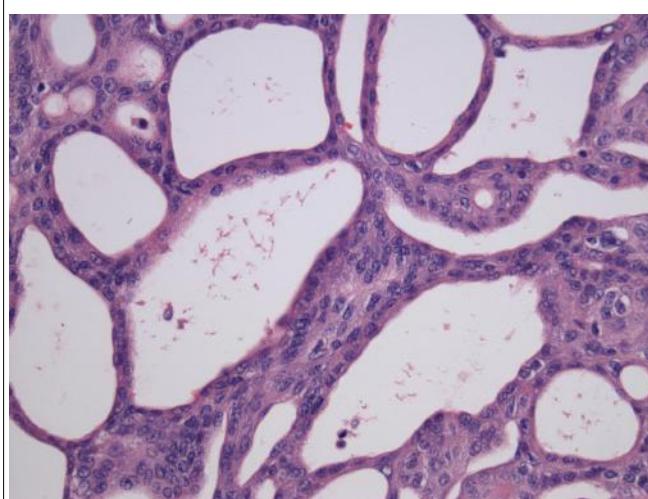


FIGURE 1 | Cribiform-morular variant of PTC showing typical cribiform arrangement composed of fused follicles lined by tall cells and lumina lacking colloid (H&E).

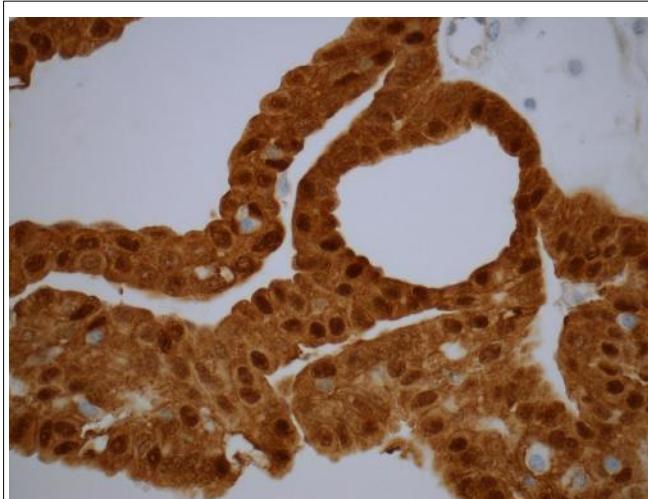


FIGURE 2 | Immunostaining for β -catenin reveals an aberrant nuclear and cytoplasmic staining in the cribiform-morular variant of papillary thyroid carcinoma. The endothelial cells are negative.

these patients had mutations in the genomic region associated with CHRPE and in the mutation cluster region in the 5' region of exon 15. This led to a recommendation that thyroid screening begin early (age 15 years) in patients or kindred with CHRPE and for patients with exon 15 mutations in the 5' region (Cetta et al., 2000).

PTEN-HAMARTOMA TUMOR SYNDROME

Cowden syndrome is an autosomal dominant disorder caused by a germline mutation in *PTEN* (phosphatase and tensin homolog, deleted on chromosome 10) and characterized by the development of multiple hamartomas and carcinomas of the thyroid, breast, and uterus. *PTEN* is a tumor suppressor gene located on 10q23.3. Two-thirds of CS patients develop thyroid tumors including multinodular goiter, multiple adenomatous nodules, follicular adenoma, follicular carcinoma and, less frequently, PTC (Parisi et al., 2001; Zambrano et al., 2004). The majority of thyroid lesions occurring in PHTS are of follicular origin and are characteristically multicentric, bilateral, benign, and malignant thyroid lesions. Adenomatous nodules seen in PHTS are bilateral, more numerous, and distinct from age-related nodules with only a scant atrophic rim of normal thyroid follicles (Nösé, 2010).

Follicular carcinoma is a major criteria and an important feature in *PTEN*-hamartoma tumor syndrome (PHTS). These tumors are more frequently multicentric, and progress from a preexisting follicular adenoma. However, PTC is rarely associated with this entity. Multiple adenomatous nodules in a background of lymphocytic thyroiditis and/or C-cell hyperplasia are distinctive findings in this syndrome. Pathologists should notify clinicians of the possibility of PHTS (Dotto and Nösé, 2008).

The diagnosis of a thyroid lesion usually precedes the diagnosis of PHTS by many years. In some patients, a work-up for PHTS is triggered by unusual pathologic findings in the thyroid of a young patient, most specifically, multiple adenomatous nodules.

The current diagnostic criteria for CS include follicular carcinoma (present in 10–15%) as a major criterion, while multinodular goiter (including multiple adenomatous nodules) and follicular adenomas are minor criteria (present in 50–67%).

Adenomatous nodules are unusually numerous in these cases reaching over 100 nodules, are not encapsulated, homogeneous, firm, yellow-tan, lack gelatinous colloid, and do not exhibit secondary changes. Histologically, the adenomatous nodules in PHTS are solid, cellular, and composed of small follicles lacking abundant colloid, side by side with others. Some nodules may have a thin, discontinuous rim of fibrous tissue simulating a capsule (Nösé, 2011). On immunohistochemical staining, 63% of CS cases showed complete loss of *PTEN* expression and 38% showed heterogeneous loss of *PTEN* (Figure 3). All intact *PTEN* expression cases were non-CS patients (Barletta et al., 2011).

The high incidence of thyroid pathology in patients with CS warrants routine thyroid screening with ultrasonography and a low threshold for recommending thyroidectomy.

CARNEY COMPLEX

The CNC is a dominantly inherited syndrome characterized by spotty skin pigmentation, endocrine overactivity, and myxomas.

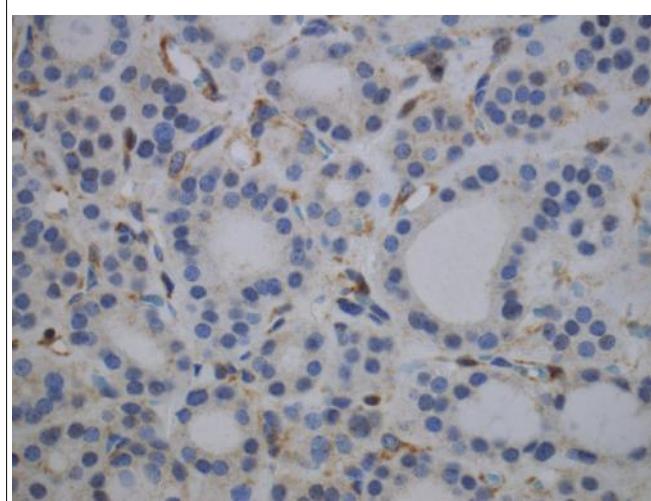


FIGURE 3 | Immunohistochemistry for PTEN shows loss of PTEN expression in an adenomatous nodule in a PTEN-hamartoma tumor syndrome (Cowden's disease) patient. Note positivity in the endothelial cells.

The syndrome was first described in 1985 as “myxomas, spotty pigmentation, and endocrine overactivity” (Carney et al., 1985).

Carney complex is defined by the association of multiple endocrine neoplasia and cutaneous manifestations. Patients previously characterized as having LAMB (lentigines, atrial myxoma, mucocutaneous myxoma, blue nevi) or NAME (nevi, atrial myxoma, myxoid neurofibroma, ephelide) could be considered to have CNC. Numerous organs may be involved in CNC and manifestations vary greatly among patients. Skin pigmentation anomalies include lentigines and blue nevi. Myxomas can occur at multiple sites such as the heart, skin or soft tissue, external auditory canal, and breast. Cardiac myxomas can develop in any cardiac chamber and may be multiple. These patients may also present with schwannomas and testicular tumors (Stratakis et al., 1997, 1998).

The most common endocrine gland involvements are growth hormone (GH)-secreting pituitary adenomas (acromegaly), thyroid tumors, and adrenocorticotrophic hormone (ACTH)-independent Cushing's syndrome due to primary pigmented nodular adrenocortical disease (PPNAD).

Most cases of this autosomal dominant condition are classified as type 1 and are associated with a mutation to the protein kinase A regulatory subunit type 1-alpha (*PRKAR1-α*) gene. Type 2 patients have been confirmed to have a mutation on chromosome 2p16, which may be a regulator of genomic stability (Stratakis et al., 1997; Matyakhina et al., 2003; Pan et al., 2010).

The risk of thyroid cancer is low, and the presence of thyroid nodules is very common. The thyroid is multinodular with multiple adenomatous nodules and follicular adenomas. Both PTC and follicular thyroid carcinomas (FTC) are present in about 15% of patients with CNC. A recent review of CNC in 53 patients of 12 kindred found clinically significant thyroid disease in 11% of patients (Stratakis et al., 1997).

WERNER SYNDROME

Werner syndrome is a rare premature aging syndrome (progeroid) that typically begins in the third decade. The clinical presentation includes an elderly appearance with thin skin, wrinkles, alopecia, and muscle atrophy in proportion to the patient's age. The patients with this syndrome have short stature secondary to an absent pubertal growth period (Nehlin et al., 2000; Muftuoglu et al., 2008). Patients also present with age-related disorders such as diabetes, osteoporosis, cataracts, peripheral vascular disease, or different types of malignant tumors. Malignancy and cardiac disease are the most common causes of death in these patients, who have a median life expectancy of 54 years (Nehlin et al., 2000; Muftuoglu et al., 2008).

It is an autosomal recessive disease that is caused by mutations in the *WRN* gene on chromosome 8p11–p12. This *WRN* gene encodes a protein that is both a RecQ helicase and exonuclease. It is important in DNA repair and replication.

Patients present at a younger age and have approximately a threefold increased risk for follicular carcinoma and six times increased risk for developing anaplastic thyroid carcinoma (Ishikawa et al., 1999).

The overall incidence of thyroid cancer in Japanese patients with Werner syndrome is 18% (Ishikawa et al., 1999). The risk of PTC is increased, specifically in Caucasian populations. This high prevalence of thyroid cancer in Werner syndrome supports routine thyroid screening in patients with this disorder.

FAMILIAL NON-MEDULLARY THYROID CARCINOMA SYNDROME

Familial non-medullary thyroid carcinoma syndrome is diagnosed when three or more family members have non-medullary thyroid cancer in the absence of other known associated syndromes. Statistical estimates suggest that a grouping of two family members with non-medullary thyroid carcinomas (NMTC) could represent the concurrence of sporadic tumors, but thyroid tumors in three or more members in kindred, or the diagnosis of PTC in men and children, is more suggestive of a familial predisposition (Malchoff et al., 1999; Musholt et al., 2000; Hemminki et al., 2005; Sturgeon and Clark, 2005; Charkes, 2006). The search for a genetic susceptibility locus for FNMTC started about a decade ago.

Familial non-medullary thyroid carcinoma is now recognized as a distinct clinical entity and accounts for up to 10.5% of all follicular cell origin thyroid carcinomas. FNMTC has been associated with the presence of multiple benign nodules, to behave in a more aggressive clinical behavior, and to have a worse prognosis than sporadic non-medullary thyroid cancer. Individuals with FNMTC have an increased risk of multifocal disease, local invasion, and increased local or regional recurrence, lymph node metastases, and intraglandular dissemination. FNMTC is an independent predictor of shorter disease-free survival (Malchoff et al., 1999; Alsanea et al., 2000; Musholt et al., 2000; Alsanea and Clark, 2001; Hemminki et al., 2005; Sturgeon and Clark, 2005; Charkes, 2006; Mazeh et al., 2012).

The genetic inheritance of FNMTC remains unknown, but it is believed to be autosomal dominant with incomplete penetrance and variable expressivity. Genetic analyses of large FNMTC kindreds not only support the hypothesis of an inherited genetic

predisposition to FNMTC, but also represent the first steps in identification of the putative susceptibility genes (Lesueur et al., 1999; McKay et al., 1999; Malchoff et al., 2000; Bevan et al., 2001; Harach, 2001; Xing, 2005). Six potential regions for harboring an FNMTC gene have been identified: MNG1 (14q32), thyroid carcinoma with oxyphilia (TCO; 19p13.2), fPTC/papillary renal neoplasia (PRN; 1q21), NMTC1 (2q21), FTEN (8p23.1–p22), and the telomere–telomerase complex. Linkage analyses have identified three different chromosomal regions that may harbor an FNMTC susceptibility gene (Nösé, 2010).

The systematic use of an algorithmic approach will help in the understanding of familial history and findings, and the patient's clinical history associated with the pathology findings to suggest a familial disease. The clinical features and relative frequency of different forms of familial thyroid cancer have been discussed and presented recently (Vriens et al., 2009).

Familial tumor syndromes characterized by a predominance of non-medullary thyroid carcinoma are listed below and summarized in Table 2.

FAMILIAL PAPILLARY THYROID CARCINOMA

Papillary thyroid carcinoma in FNMTC has a well-documented predisposition to multicentric disease, bilateral disease, local invasion, extrathyroidal extension, lymph node metastases, recurrence, and specific histology.

The background thyroid may show lymphocytic thyroiditis, multinodular hyperplasia, and multiple adenomatous nodules. Sporadic PTC has *BRAF* mutation in approximately 40% of cases. However, no *BRAF* mutation was reported in a group of 40 patients with FNMTC as germline mutation or a susceptibility genetic event for FNMTC (Xing, 2005).

The fPTC enriched in TCO has been mapped to chromosomal region 19p13, and FNMTC without oxyphilia has also been mapped to 19p13.31. Tumor-specific loss of heterozygosity is found in sporadic FTC with and without oxyphilia at both 19p13 and 2q21.32 (Lesueur et al., 1999; Musholt et al., 2000).

FNMTC TYPE 1 SYNDROME

The FNMTC type 1 syndrome (chromosomal region 2q21) is characterized by PTC without any distinguishing pathologic features

Table 2 | Familial tumor syndrome characterized by a predominance of non-medullary thyroid carcinoma.

Tumor type	Inheritance	Chromosomal loci	Candidate genes
PTC associated with PRN	Unknown	1q21	Unknown
Familial MNG with PTC	Autosomal dominant	14q	Unknown
Familial PTC	Unknown	2q21	Unknown
Familial TCO and without oxyphilia	Autosomal dominant	19p13.2	Unknown/ TCO/T1MM44

PRN, papillary renal cell neoplasia; PTC, papillary thyroid cancer; MNG, multinodular goiter; TCO, thyroid carcinoma with oxyphilia.

and without an obvious increase in frequency of non-thyroidal neoplasms in kindred members (McKay et al., 2001).

fPTC ASSOCIATED WITH RENAL PAPILLARY NEOPLASIA

The fPTC associated with renal papillary neoplasia presents with the usual classical variant of PTC and with no special features. The PRN syndrome (fPTC/PRN), mapped to chromosomal region 1q21, includes not only PTC and the expected benign thyroid nodules, but also PRN and possibly other malignancies as well (Malchoff et al., 2000).

FAMILIAL MULTINODULAR GOITER SYNDROME

In familial multinodular goiter syndrome, which is mapped to 14q, some patients may develop an associated PTC (Bakhsh et al., 2006).

CONCLUSION

Mutations in patients with FNMTC syndromes have not been well defined as in medullary thyroid carcinoma (MTC). Most patients

with these familial syndromes may have susceptibility genes that increase the risk of thyroid cancer. Thyroid cancer in many of these patients has been characterized as more aggressive than sporadic thyroid cancer, with a predisposition for lymph node metastasis, extrathyroidal invasion, and a younger age of onset. Most patients with a familial syndrome and FNMTC will have PTC, suggesting that a specific gene for PTC may also be present. In many cases, patients have a known familial syndrome that has a defined risk for thyroid cancer. These patients can be followed closely and the thyroid can be identified at an earlier stage (Nösé, 2010). In a few cases, thyroid cancer may be the initial presentation of an underlying familial syndrome.

The distinct thyroid pathology in some of these syndromes should alert the pathologist of a possible familial cancer syndrome. Knowledge of FNMTC and its histopathology can allow the pathologist to diagnose familial tumor syndrome. Awareness and screening of FNMTC will permit earlier detection, proper treatment, and improved outcomes for patients and their families.

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Rethinking the role of oncogenes in papillary thyroid cancer initiation

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Thyroid cancer originating from follicular epithelial cells accounts for approximately 1% of all new cases of cancer each year and its incidence has increased significantly over the last two decades (Hodgson et al., 2004; Davies and Welch, 2006). Papillary thyroid carcinoma (PTC) accounts for approximately 85% of all cases, and it is responsible for the overall increase in incidence of thyroid cancer. Mortality in PTC is low and the majority of patients can be considered cured after thyroidectomy followed by ablation of thyroid remnant by 131-iodine (Cooper et al., 2009).

Molecular studies performed in the last decades, have elucidated in part the molecular mechanisms underlying thyroid cancer initiation and progression. Specific genetic alterations are associated to this thyroid tumor histotype: *RET/PTC* and *TRK* rearrangements and *BRAF* and *RAS* mutations.

The first genetic alteration discovered in PTC and also the most specific was the *RET/PTC* rearrangement (Fusco et al., 1987). *RET/PTC* is a chimeric gene generated by the fusion of the tyrosine kinase domain of the rearranged during transfection gene (*RET*) to the 5' terminal region of genes that are constitutively expressed in thyroid follicular cells (Pierotti et al., 1992; Santoro et al., 1992, 2006; Nikiforov, 2002). The chimeric proteins generated dimerize in a ligand-independent manner and result in a cytoplasmatic constitutively active tyrosine kinase. The higher frequency of PTC observed in the population exposed to the Chernobyl accident supports a role for the external radiations in the chromosome rearrangements observed in this tumor (Nikiforov, 2006). It has been proposed that the spatial proximity of translocation-prone gene loci may favor gene rearrangements. Indeed, proximity between *RET* and *H4*, and *NTRK1* and *TPR* has been

reported in interphase thyroid nuclei. Thus, in this simplified model, radiations induce chromosome rearrangements and generation of *RET/PTC* or *TRK* oncogenes that will be initiator of thyroid carcinogenesis. The role of *RET/PTC* in thyroid carcinogenesis is supported by experimental evidences generated in cells in culture and in animal models. PCC13, a differentiated rat thyroid cell line, stably transfected with a *RET/PTC3* expressing plasmid undergoes morphological alterations and is no longer TSH dependent for growth (Santoro et al., 1993). Thyroid-specific expression of the *RET/PTC1* or *RET/PTC3* in transgenic mice induces thyroid tumors with features resembling those of human PTC. These tumors are characterized by nuclear grooves and ground glass cells, continuous slow growth rate, and loss of iodide uptake (Jhiang et al., 1996; Santoro et al., 1996). However, some evidence suggest that *RET/PTC* alone is not sufficient to develop thyroid carcinoma, and other molecular events are needed. Thyroid cancer occurs only after a long latency period and only in a fraction of *RET/PTC* transgenic animals. At beginning, the majority of studies excluded the occurrence of *RET/PTC* in benign thyroid nodules. In following studies, *RET* rearrangements have been demonstrated in nodules diagnosed as benign at histology. Ishizaka et al. (1991) have been the first to detect *RET/PTC* in 21% of follicular adenomas. The use of highly sensitive detection methods contributed to definitively demonstrate that *RET* rearrangements occurs in a significant fraction of both radiation-induced and sporadic benign nodules (Bounacer et al., 1997; Cinti et al., 2000; Guerra et al., 2011; Marotta et al., 2011a; Sapiro et al., 2011). Its presence in benign nodules, raised some queries about the role of *RET/PTC* in thyroid carcinogenesis.

Doubts on the primary role of *RET/PTC* in thyroid carcinogenesis are also supported by the evidence that some irradiated PTC are composed of a mixture of cells with and without *RET* rearrangements. In sporadic microcarcinomas and post-Chernobyl PTC interphase fluorescence *in situ* hybridization (FISH) analysis demonstrated that *RET/PTC* rearrangements can occur only in a fraction of the cells, indicating that PTC can be composed of a mixture of cells with and without *RET* rearrangements (Corvi et al., 2001; Unger et al., 2004). These evidences are in favor of a secondary role of *RET/PTC* which would not be the initiating event in thyroid carcinogenesis.

BRAF is a protein-serine/threonine kinases that participate in the mitogen-activated protein kinase (MAPK) cascade (Wellbrock et al., 2004). By modulating the MAPK cascade, *BRAF* plays a pivotal role in many aspects of cell biology in nearly every cell type. More than 65 different mis-sense *BRAF* mutations have been detected in human cancer so far (Davies et al., 2002). The *BRAF^{V600E}* mutation, resulting from the *BRAF^{T1799A}* transversion, is nearly the only mutation of this kinase found in thyroid cancer and the most common genetic mutation in PTC, being detected in about 50% of cases (Kimura et al., 2003; Xing, 2005; Marotta et al., 2011b). This mutation occurring within the activation segment, disrupts the hydrophobic interaction between the glycine-rich loop of the N-terminal region and the activation segment of the kinase domain, and transforms *BRAF* in a constitutively activated kinase (Davies, et al., 2002; Brummer et al., 2006; Moretti et al., 2009). In the thyroid, this oncogene is restricted to papillary-patterned cancer and it does not occur in Hashimoto's thyroiditis, benign colloid nodules, thyroid adenomas, or other types of thyroid tumor (Xing, 2007).

Its restricted occurrence makes *BRAF^{V600E}* of clinical diagnostic utility (Xing, 2007; Zatelli et al., 2009). Its carcinogenic potential has been demonstrated in several different cell types and in animal models. Thyroid-specific expression of *BRAF^{V600E}* obtained in transgenic mice by the bovine thyroglobulin promoter provided us with important information on the tumorigenic potential of this oncogene. By 12–22 weeks age, transgenic mice revealed a large goiter, well differentiated thyroid cancer foci, and poorly differentiated foci in some animals, depending on the level of expression of the *BRAF^{V600E}* mRNA. These tumors displayed a phenotype similar to that one of spontaneous human PTC, supporting a key role for this oncogene in the tumor initiation of this type of cancer and in the progression to poorly differentiated carcinomas (Knauf et al., 2005). In a more recent animal model, expression of *BRAF^{V600E}* was obtained in adult mice in already developed thyroid glands. After 1 month of induced expression of the oncogene, mice developed an hypercellular thyroid, up to 10 times larger in size than controls, whilst nodules of tumor cells displaying a characteristic papillary structure were readily apparent 6 months after *BRAF^{V600E}* expression (Charles et al., 2011). These experimental animal models demonstrate that *BRAF^{V600E}* can promote the transformation process of the thyroid follicular cell, however they do not demonstrate that this oncogene is the initiating event in spontaneous human PTC. Very recently a more detailed analysis of *BRAF^{V600E}* expression by means of a quantitative assay, demonstrated the heterogeneous intratumoral nature of spontaneous PTC. The analysis of the percentage of mutant *BRAF* demonstrated that clonal *BRAF^{V600E}* is a rare occurrence in PTC, while more frequently this cancer consists of a mixture of tumor cells with wild-type and mutant *BRAF*. This result demonstrates that *BRAF* mutation in PTC is a secondary subclonal event (Guerra et al., 2012a).

Thus, the original idea that a normal thyroid cell, under the effect of ionizing radiations or other mutagenic factors, acquires the *RET/PTC* rearrangement or *BRAF^{V600E}* mutation and consequently is transformed by these oncogenes in what we call a PTC cell, should be revised. Although heterogeneity is the rule in cancer, the identification of the genetic initiating event is important, not only to understand the molecular

mechanisms of tumorigenesis, but also for practical purposes. A high percentage of *BRAF^{V600E}* alleles is associated with a higher frequency of recurrence (Guerra et al., 2012b). This makes a quantitative assessment necessary to use *BRAF^{V600E}* in clinical practice as a predictor of recurrence in PTC. Also targeted therapeutic interventions must take into account of this heterogeneity. The recent development of novel small-molecule inhibitors targeting one or more of these oncogenes may provide selective advantages for the treatment of advanced thyroid cancer harboring these mutations (Salerno et al., 2010; Nucera et al., 2011). Many of these promising drugs are currently being evaluated in clinical trials and the presence of target-negative subpopulations should be considered.

In conclusion, we have to revise our simplistic vision of thyroid carcinogenesis. Oncogenes known so far may play important role in the fate of a PTC, conferring specific biological and clinical features, but the genetic event initiating thyroid cancer is still to be identified.

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Application of liquid-based cytology to fine-needle aspiration biopsies of the thyroid gland

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Fine-needle aspiration biopsy is regarded as an important tool for diagnosing thyroid lesions because of its simplicity, safety, and cost-effectiveness. Its role in correctly characterizing the group of indeterminate lesions or follicular-patterned neoplasms (FN) might be more decisive. Liquid-based cytology (LBC) is a technique based on the use of a semi-automated device that has gained popularity as a method of collecting and processing both gynecologic and non-gynecologic cytologic specimens. It achieves a diagnostic sensitivity as accurate as conventional preparations especially for its excellent cell preservation and for the lack of background which decrease the amount of inadequate diagnoses. Moreover, the cellular material which has been stored in the preservative solution could be effectively used for the application of immunocytochemical and molecular techniques especially for the Follicular proliferations. In many cases the cytologic features are similar in both methods but the colloid film and the lymphocytic component are more easily evaluated on direct smears whereas nuclear details and colloid globules are better evaluated in LBC slides. The LBC-processed biopsies represent a valid alternative to conventional cytology. The possibility of applying special techniques enhance the efficacy of the cytological diagnosis of thyroid lesions.

Keywords: thyroid nodules, fine-needle aspiration cytology, liquid-based cytology

INTRODUCTION

Fine-needle aspiration biopsy (FNAB) represents an invaluable diagnostic tool for characterizing thyroid nodules with a worldwide consensus for its simplicity, safety (Galera-Davidson and Gonzalez-Campora, 2008), and regarded as the most accurate and cost-effective method for the selection of surgical patients.

The liquid-based cytology (LBC) technique, originally developed for application to gynecologic cervical smears, has progressively gained consensus for both non-gynecologic and fine-needle aspiration cytological material (Biscotti et al., 1995; Rossi and Fadda, 2008; Rossi et al., 2009).

This method is based on a two-step procedure: (1) the fixation of the material in an methanol-based solution and (2) the automated processing of the material to obtain a thin layer of cells with a computer-assisted device. The two most common methods for processing the cytologic samples are: ThinPrep2000TM (Hologic Co., Marlborough, MA, USA), in which the cells are aspirated from a methanol-based solution (CytolytTM) then filtered and transferred onto a positively charged slide with a gentle positive pressure; in the method SurePathTM (TriPath Imaging, Burlington, NC, USA) the cells are collected in an ethanol-based solution (CytoRichTM), centrifuged twice then slowly sedimentated onto a poly-L-lysinated slide and eventually stained with a specific hematoxylin–eosin stain. The final result for both methods is one slide for each lesion with all cells concentrated in the central area of

the slide with a sensitivity of 77% and a specificity of 81% (Geers and Bourgoin, 2011).

Despite the initial controversy regarding the efficacy of the use of Thin Prep alone (Cochand-Priollet et al., 2003; Fadda et al., 2006; Rossi et al., 2010) good results have been achieved by many groups in different countries, especially in the recent years. Since November 2003 until 2011 the majority of about 22,000 FNABs carried out in the "Agostino Gemelli" School of Medicine and Hospital of Rome have been processed by ThinPrep2000TM alone. This experience has been reported in many studies published since 2005 where the efficacy of the ThinPrep2000TM technique for a correct pre-operative diagnosis of more than 500 malignant neoplasms is highlighted. In the study by Rossi et al. (2009), three parameters of efficacy (inadequacy, indeterminacy, and malignancy rates) were chosen for evaluating the efficacy of ThinPrep2000TM in comparison with CS alone and combining ThinPrep2000TM and CS in more than 10,000 thyroid FNAB showing that ThinPrep2000TM alone was as effective as CS in decreasing both inadequate and indeterminate diagnoses (Fadda et al., 2011b). The study of Geers and Bourgoin (2011) using the SurePath method achieves controversial results in terms of inadequacy rate between LBC and SurePath.

The ThinPrep2000TM material stored in the vial can be used for additional techniques such as immunocytochemistry, flow cytometry, and molecular biology (Cochand-Priollet et al., 2003; Rossi et al., 2005).

CYTOLGY OF LBC-PROCESSED THYROID LESIONS

BENIGN LESIONS

The morphologic picture of LBC differs mainly from CS in two aspects: (a) the cells in each slide are a monolayered representative sample of the entire material collected in the vial with a variable amount of cells which remains in the preservative solution; (b) the automated process causes some changes in both cellular and background morphology. One of the most important change, occurring in LBC slides, is the appearance of the fragmented colloid which is present as small droplets in the background of a benign nodule with a quantitative detection whereas in CS the colloid usually does not require a quantization (Figure 1; Biscotti et al., 1995). The LBC picture of a thyroiditis is similar to CS with the exception of the amount of lymphocytes in the background which can be higher than normal because of the spinning of the material before the automated process. When a thyroiditis is suspected, the detection of lympho-epithelial clusters in an inflammatory background is the pivotal clue for the diagnosis and warrants a simple follow-up for the patient (Das, 2006).

FOLLICULAR-PATTERED LESIONS

There are very few differences in the cytologic pictures of follicular neoplasms (FN) in LBC versus CS based upon the identification of microfollicles made up of medium-sized thyrocytes in scant colloid. The amount and morphology of the follicular cells allow the inclusion of an individual lesion in one of the categories that have been recently devised in Europe and in the USA with three possible scenario (British Thyroid Association, 2007; Baloch et al., 2008; Fadda et al., 2010):

(1) The first scenario is represented by a lesion which high cellularity but the cells are monomorphic with occasional enlarged nuclei. This lesion may correspond to the cellular adenomatous nodule and is usually included in the non-neoplastic category of the European classifications (Thy 2 by

BTA classification, TIR 2 by Italian classification,). On the other hand, the Bethesda classification has established a different category for this picture which is defined as “follicular lesion with undetermined significance (FLUS)” or “atypical cells of undetermined significance (ACUS or AUS)” with a different risk of malignant occurrence which in the American classification is stated between 5 and 15% (mostly follicular carcinoma) whereas in the European systems is closer to non-neoplastic lesions (British Thyroid Association, 2007; Baloch et al., 2008; Fadda et al., 2010).

- (2) The second scenario is represented by a lesion mostly follicular-structured and made up of medium-sized thyrocytes with rounded nuclei and central nucleolus (Follicular Neoplasm) with a malignancy risk between 20 and 30%. The action, although debated in the literature, results in the surgical removal of the lesion which could histologically correspond to both a follicular adenoma or an adenomatous nodule in a goiter (70–80% of cases) but also follicular carcinoma or a follicular variant of a papillary carcinoma (PC) cannot be ruled out only on morphology. The same diagnostic criteria and therapeutic action is applied to the FN composed mostly by oxyphilic (or Hurthle) cells which is defined when follicles are made up of more than 80% of oxyphilic cells and it should be included in the FN category (Thy 3 of the BTA, TIR 3 of the Italian classifications). The colloid amount may be scant (but sometimes is abundant) and features of old hemorrhage (hemosiderin-laden histiocytes) may coexist (Giorgadze et al., 2004; British Thyroid Association, 2007; Baloch et al., 2008).
- (3) The third scenario is represented by follicular-structured lesions, composed by thyrocytes with elongated and clear nuclei, sometimes with grooves and peripheral nucleoli without papillae, psammomatous bodies, or nuclear pseudo inclusions with a risk of malignancy ranging between 50 and 70%. This category warrants the surgical removal of the nodule as a follicular variant of a PC is very likely to be found at the histological examination (more than 90% of cases).

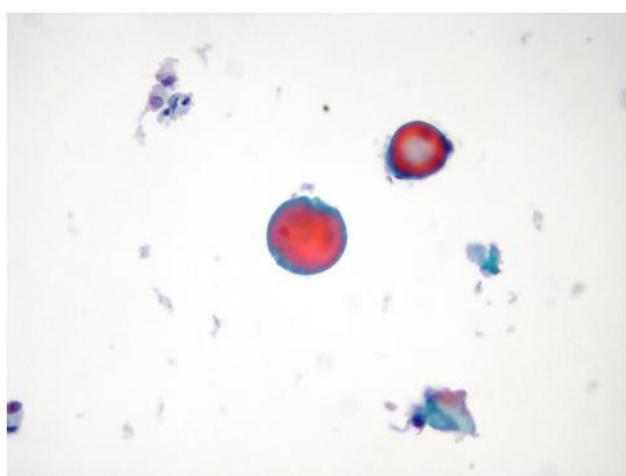


FIGURE 1 | A case of colloid goiter exhibiting small clusters of monomorphic follicular cells mixed with colloid droplets (LBC, Papanicolaou, 400x).

MALIGNANT TUMORS

The cytological diagnosis of thyroid malignancy does not differ substantially in LBC preparations as the clear background facilitates the identification and characterization of the cellular details.

The most important malignant tumor which can be appropriately identified on LBC preparations is PC which is easily when nuclear pseudo inclusions are detected (Figure 2) papillary structures and psammoma bodies are seldom identified. In the earlier reports, the difficulty in detecting the distinctive nuclear features of PC was one of the most important objections against the adoption of the thyroid LBC cytology.

Medullary thyroid carcinoma (MTC) is a difficult cytological diagnosis. The LBC technique offers the opportunity to detect the calcitonin expression in the neoplastic parafollicular cells and their concomitant negativity for thyroglobulin (Rossi et al., 2008).

Anaplastic thyroid carcinoma (ATC) is seldom seen in routine thyroid cytology. The LBC picture of ATC usually shows a background of necrotic debris with small clusters of large round or spindle cells with pleomorphic nuclei and prominent nucleoli which stains positive for cytokeratins (useful to confirm the

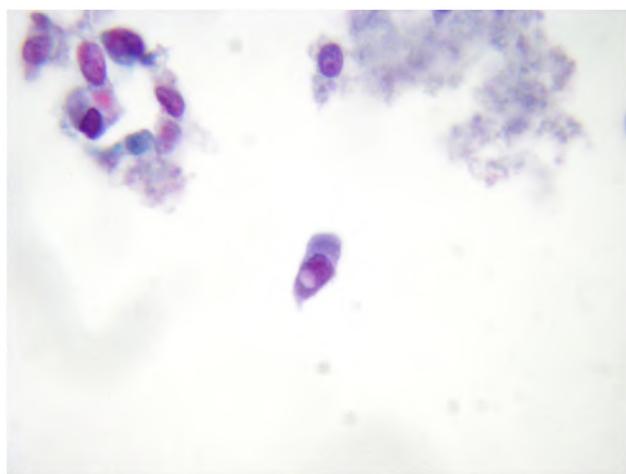


FIGURE 2 | A distinctive nuclear pseudoinclusion in a fine-needle aspiration biopsy from a thyroid papillary carcinoma (LBC, Papanicolaou, 400x).

epithelial origin) and negative for thyroglobulin and TTF-1. The LBC diagnosis of the large cell variant of malignant non-Hodgkin lymphoma usually does not constitute a problem and relies on the immunocytochemical expression of LCA, CD20, bcl-6, and other lymphoid antigens.

Lung, breast, kidney, large bowel, and laryngeal metastatic carcinomas to the thyroid gland may occasionally present as a single nodule mimicking a primary tumor in which necrotic debris or hemorrhagic material and clusters of neoplastic cells with features of adenocarcinoma or squamous cell carcinoma are detected on Cs and ThinPrep2000TM

SPECIAL TECHNIQUES

IMMUNOCYTOCHEMISTRY

Immunocytochemistry was introduced in the early 1970s for the definition of the nature of the lesions, especially in the differential diagnosis between follicular and C-cell derived neoplasms and in the identification of primary or metastatic thyroid neoplasms (e.g., malignant lymphoma) even if the main role is the identification of the markers of malignancy which may distinguish malignant from benign lesions regardless of the presence of capsular and vascular invasion (Rossi et al., 2006).

There have been only few reported experiences in literature dealing with ICC applied on ThinPrep2000TM. The use of ICC on the cells stored in the preservative ThinPrep2000TM solution yields excellent results with most immunoreagents in terms of staining pattern, intensity of the reaction and less amount of reagent due to the clear background and smaller size of the ThinPrep2000TM slide (Dabbs et al., 1997; Leung and Bedard, 1999; Rossi et al., 2006).

In our practice and based on the data in the literature, no “magic single marker” may be useful but only the concordance of a panel should be considered especially in cases of follicular lesions. The use of more than one immunomarker is a further guarantee for a correct diagnostic approach, especially when a concordant panel is expressed (Rossi et al., 2006) and with excellent results also in the differential diagnosis between benign and malignant follicular

neoplasms (Dabbs et al., 1997; Leung and Bedard, 1999; Schmitt et al., 2008; Fadda et al., 2011a).

HBME-1, Galectin-3, and RET proto-oncogene have shown the best specificity and sensitivity in discriminating benign from malignant differentiated tumors. These data emerged from one of the paper of our group, in which the combination of nuclear pleomorphism and positivity of the panel resulted in 75% specificity and 89% diagnostic accuracy of FNAB (Rossi et al., 2005). In another study recently published by our group, the complete immunocytochemical panel (made up of HBME-1 and Galectin-3) was positive in 83.3% of malignancy and negative in 87.5% benign histological cases. In the group of FN/AUS (according to the Bethesda system), the expression of HBME-1 and Galectin-3 on LBC can effectively distinguish lesions which need immediate surgery (high risk FN) from those which can be followed-up (low risk FN).

MOLECULAR TECHNIQUES

Recent advances in molecular genetics of thyroid cancer are being applied for developing new diagnostic markers for FNA samples in an attempt to differentiate benign from malignant thyroid nodules (Nikiforova and Nikiforov, 2009). PC, the most common thyroid malignancy, may carry BRAF, Ret/PTC, or RAS mutations (Nikiforova and Nikiforov, 2009). These mutually exclusive somatic mutations are found in more than 70% of PCs with a more aggressive tumor behavior such as extra-thyroidal extension, advanced tumor stage at presentation and lymph node or distant metastases (Xing, 2007).

Several studies have demonstrated the feasibility of detecting BRAF, RET/PTC, or RAS mutation in thyroid FNA samples and have shown that this may improve the cytological FNA diagnosis (Nikiforova and Nikiforov, 2009; Ohori et al., 2010; Fadda et al., 2011b).

A recent paper by Nikiforova and Nikiforov (2009) explored the diagnostic utility of molecular testing for a panel of molecular mutations consisting on BRAF, RAS, RET/PTC, and PAX8-PPAR γ in 480 FNA samples from thyroid nodules which were prospectively tested and yielded 32 mutations with 31 malignant surgical diagnoses and only one case of follicular adenoma.

The possible diagnostic use of molecular markers is reflected in the last guidelines published by the American Thyroid Association. These guidelines indicate that the use of molecular markers such as BRAF, RAS, RET/PTC, and PAX8-PPAR γ may be considered (with low recommendation rate) for patients with indeterminate FNA cytology to help guide their clinical management.

As resulted in our preliminary study the evaluation of BRAF positivity could be a prognostic value for the presence of nodal metastases and the presence of BRAF positivity performances to the surgical neck central dissection and also to the detection of any other nodal possible implication (Fadda et al., 2011b).

Liquid-based cytology can offer the possibility of immunocytochemical evaluation and molecular testings for common somatic mutations in thyroid FNAB as the nucleic acids are stable in the preservative solution up to 6 months after the sampling. In this setting, the future possibility of a guideline encompassing the combined use of immunocytochemistry and molecular tests for supplementing the morphologic diagnosis could be the starting point for a complete pre-operative assessment of a thyroid lesion.

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Anaplastic thyroid carcinoma

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Thyroid cancers represent about 1% of all human cancers. Differentiate thyroid carcinomas (DTCs), papillary and follicular cancers, are the most frequent forms, instead Anaplastic Thyroid Carcinoma (ATC) is estimated to comprise 1–2% of thyroid malignancies and it accounts for 14–39% of thyroid cancer deaths. The annual incidence of ATC is about one to two cases/million, with the overall incidence being higher in Europe (and area of endemic goiter) than in USA. ATC has a more complex genotype than DTCs, with chromosomal aberrations present in 85–100% of cases. A small number of gene mutations have been identified, and there appears to be a progression in mutations acquired during dedifferentiation. The mean survival time is around 6 months from diagnosis an outcome that is frequently not altered by treatment. ATC presents with a rapidly growing fixed and hard neck mass, often metastatic local lymph nodes appreciable on examination and/or vocal paralysis. Symptoms may reflect rapid growth of tumor with local invasion and/or compression. The majority of patients with ATC die from aggressive local regional disease, primarily from upper airway respiratory failure. For this reason, aggressive local therapy is indicated in all patients who can tolerate it. Although rarely possible, complete surgical resection gives the best chance of long-term control and improved survival. Therapy options include surgery, external beam radiation therapy, tracheostomy, chemotherapy, and investigational clinical trials. Multimodal or combination therapy should be useful. In fact, surgical debulking of local tumor, combined with external beam radiation therapy and chemotherapy as neoadjuvant (before surgery) or adjuvant (after surgery) therapy, may prevent death from local airway obstruction and as best may slight prolong survival. Investigational clinical trials in phase I or in phase II are actually in running and they include anti-angiogenetic drugs, multi-kinase inhibitor drugs.

Keywords: anaplastic, cancer, genetic alteration, prognosis, therapy, thyroid, treatment, tumor

EPIDEMIOLOGY

The majority of thyroid malignancies are well-differentiated and have an excellent prognosis, on the other hand Anaplastic Thyroid Carcinoma (ATC) is an extremely aggressive cancer representing one of the most aggressive in humans with a dismal prognosis despite various therapeutic modalities. Although ATC accounts only for 2% of thyroid cancer incidence, it accounts for 14–39% of thyroid cancer deaths (Hundahl et al., 1998; Kitamura et al., 1999).

The female/male ratio is 5 to 1 and the peak of incidence is in the sixth and seventh decades of life (Kebebew et al., 2005). These observations are confirmed by other studies have demonstrated that about 68% of ATC patients were over 70 years of age and females constituted 70% and males 30% (Hundahl et al., 2000). The incidence of ATC is estimated at one to two cases per million population per year, and the trend has been decreasing even though the incidence of well-differentiated subtypes (e.g., papillary and follicular) of thyroid cancer has been increasing (Davies and Welch, 2006).

RISK FACTOR

Risk factors for ATC are not well understood, in fact ATC might occur in patient without history of thyroid disease, or on the other

hand, could have a history of goiter, or on histological examination, could co-exist differentiated thyroid cancer. In a study was demonstrated that 25% of the ATC patients had a prior history of thyroid goiter and another 10% with family history of goiter and it is also known that ATC is more common in places with endemic goiter as demonstrated by decreased incidence of ATC after iodine salt supplementation (Besic et al., 2010).

GENETIC ALTERATIONS

Recent advances in understanding the genetic and molecular pathogenesis of ATC hold promise for target therapy. ATC shows only few specific gene mutations, and the majority occurs in PTC (e.g., RAS and BRAF; Smallridge et al., 2009). This implies that many ATCs derive from preexisting PTCs by a process of dedifferentiation, acquiring new mutations like p53, catenin (cadherin-associated protein), beta 1, and PIK3CA (Nuocera et al., 2011). The remaining part of ATC may derive “*de novo*,” in this evenuality ATC is found alone in the removed thyroid (Nikiforov, 2004).

Genetic alterations in p53 gene are the most frequent in ATCs (55%), the other mutations follow for frequency as reported: RAS (22%), BRAF (26%), b-catenin (38%), PIK3CA (17%; Smallridge and Copland, 2010).

p53 GENE

It is a tumor suppressor gene located on chromosome 17p. P53 plays a key role in its nuclear transcription of factor production and in regulation of the cell cycle, DNA repair, and apoptosis. Mutations of p53 gene result in growth, angiogenesis, and dedifferentiation (O'Neill et al., 2010). Antico Arciuch et al. (2011) recently presented a paper with the characterization of the first mouse model of ATC, derived from the inactivation of p53 in cavies with constitutional activation of PI3CA.

BRAF

It is the most common mutation in papillary thyroid cancer and it is involved in tumor progression like radioactive iodine refractoriness and PTC recurrence (Nucera et al., 2010). BRAF mutations can be observed in both differentiated and undifferentiated tissues when ATCs co-exist with PTCs (Begum et al., 2004; Soares et al., 2004).

RAS

It is a family of oncogenes important as cell growth regulators and they have a role in thyroid differentiation (Santarpia et al., 2008). The most important signal transduction pathway, for tumorigenesis, is the Ras-MAPK and phosphatidylinositol 3-kinase (PI3K)/Akt pathways (Luo et al., 2003).

***b*-CATENIN AND Wnt PATHWAYS GENES**

b-Catenin is a multifunctional protein and has two distinct functions. At first it binds as an intracellular stabilizer to cadherins to form the adherence junction. Dissociation of *b*-catenin from E-cadherin, mediated by various TKs, favors cell migration, and formation of metastases. At second, activation of Wnt signaling pathway stabilizes cytosolic *b*-catenin. This mechanism could be lost in presence of *b*-catenin mutations (Rao et al., 2006).

PIK3CA

Among PI3Ks there are several classes, but the most important in human tumorigenesis and it is also the well characterized. It consists of heterodimers of a regulatory subunit and one of p110 catalytic subunits. The A and B types (respectively PIK3CA and PIK3CB) play an important role in human cancers (Xing, 2010). PIK3CA gene is located on 3q26.3 and its mutations are extremely rare in follicular or papillary thyroid carcinomas (Liu et al., 2008).

CLINICAL PRESENTATION

Clinically, ATC can present as a rapidly progressing disease invading surrounding local tissues and metastasizes to distant organs. Locally, ATC shows a rapidly enlarging anterior neck mass, with accompanying dysphagia (40%), voice change or hoarseness (40%), and stridor (24%). Regional symptoms included a noticeable lymph node mass (54%) and neck pain (26%). Systemic symptoms include anorexia, weight loss, and shortness of breath with pulmonary metastases. ATC is usually advanced at diagnosis and frequently surgically unresectable (Kebebew et al., 2005; Besic et al., 2010). Around 20–50% of patients present with distant metastases, most often pulmonary (Nuocera et al., 2011), and another 25% develop new metastasis during the rapid course of the disease [Lungs (80%), bone (6–16%), and brain (5–13%) were the most common sites of metastasis; Tennvall et al., 2002].

PROGNOSIS

The three different morphologic patterns identifiable at histologic analysis (squamous, spindle cell, and giant cell) present similar biological and clinical features and none influences the prognosis (Hundahl et al., 1998).

Some factors as age, gender, tumor size, extent of disease, and resectability, influence clinical course and prognosis, however the median survival is usually less than 6 months, and death is either due to uncontrolled local invasion or distant metastasis. Younger female patients (<65 years old), with a small ATC (less than 5 cm or intra-thyroidal) and no distant metastasis at diagnosis, have a better prognosis (Rosai et al., 1993). Local disease consists in tracheal and esophageal invasion and obstruction (Paszek, 2003; Kihara et al., 2004).

In an analysis of survival of ATC patients from the SEER database from 1983 to 2002, which included patients who survived for more than a month, the median survival was 4 months. On multivariate analysis, distant, or metastatic disease, tumor size greater than 7 cm, and treatment with surgery with or without radiotherapy were statistically significant prognostic markers with poor outcomes ($P \leq 0.05$). Of interest, patients with extracapsular extension into adjacent tissue, the addition of radiotherapy was of benefit, on the contrary radiotherapy after surgery was of no benefit in patients who had disease confined to the thyroid or had distant metastasis (Sniezek, 2003). Age, sex, size of the tumor, resectability, and the extent of disease has been shown to affect the course of the disease (Chen et al., 2008). In a SEER-based study in the United States by multivariate analysis, only age less than 60 years, an intra-thyroidal tumor, and the combined use of surgical and external beam radiation therapy were identified as independent predictors of lower cause-specific mortality (McIver et al., 2001). In other series, female sex, tumor size less than 6 cm, age, and the extent of disease were the most favorable prognostic markers (Tan et al., 1995; Dziba et al., 2002). Among Koreans less than 60 years of age, tumor size less than 7 cm, and lesser disease burden were independent predictors of lower mortality (Lo et al., 1999). A recent study from France based on 26 patients with ATC, univariate analysis showed that age above 75, capsular invasion, lymph nodes metastasis, residual tumor after surgery, and lack of multimodal treatment (particularly radiotherapy in patients without tumor residue) are poor prognostic factors. Multivariate analysis in the same cohort showed age above 75, followed by node invasion, capsular invasion, and female sex to be poor prognosticators (Roche et al., 2010). In a study by Venkatesh and colleagues, patients with localized disease had a median survival of 8 months in comparison to 3 months for patients with metastatic disease (Tan et al., 1995; Lo et al., 1999; Kim et al., 2007; Roche et al., 2010). A prognostic index was developed by Sugitani et al. (2001) from a review of their series of 47 patients over 33 years (Venkatesh et al., 1990). Their index was based on a combination of four risk factors: (Hundahl et al., 1998; Kitamura et al., 1999) presence of acute symptoms, (Kebebew et al., 2005) tumor size greater than 5 cm, (Hundahl et al., 2000) distant metastasis, and (Davies and Welch, 2006) white cell count $\geq 10,000/\mu\text{L}$ (Venkatesh et al., 1990). Patients with a prognostic index less than or equal to one had a 62% survival rate at 6 months, whereas all patients with prognostic index of three and four died within 6 and 3 months, respectively.

TREATMENT

Patients with ATC even in the absence of metastatic disease are considered to have systemic disease at the time of diagnosis. All ATCs are considered stage IV by the International Union Against Cancer (UICC) – TNM staging and American Joint Commission on Cancer (AJCC) system. Multimodality treatment consisting of surgery when feasible combined with radiation and chemotherapy is generally recommended.

SURGERY

The aim of surgery is to obtain a complete macroscopic resection, with microscopically clear resection margins. Complete resection has been identified as a prognostic factor in several clinical trials (Junor et al., 1992; Tan et al., 1995; Kobayashi et al., 1996; Sugitani et al., 2001). When feasible, surgery must aim at a radical intent. The categories of patients that may be most suitable for this approach are young patients (<65 years old) with small lesions (<6 cm) and no distant metastasis. However, surgery also plays an important role for palliation. Partial resection of the tumor followed by radiotherapy and chemotherapy may delay or avoid airway obstruction, although it can improve survival only by a few months (Nel et al., 1985). It is theoretically possible that, in selected patients, even in the setting of metastatic disease, surgery may result in an improved quality of life and prevent death from suffocation (Miccoli et al., 2007).

Since surgery alone is not able to control the disease even in patients with small intra-thyroidal masses, adjuvant therapy is always required, and can be administered either with radiotherapy (RT) or chemoradiotherapy. Whether surgery should be given up-front or after neoadjuvant treatment is a matter of debate. In fact, primary chemotherapy might make inoperable lesions operable, with the additional potential advantage of preventing distant metastasis. Moreover, Tennvall et al. (2002) reported encouraging results analyzing the outcome of 55 patients with ATC treated with neoadjuvant chemoradiotherapy between 1984 and 1999. The response to primary treatment was 72% (Yau et al., 2008).

SYSTEMIC TREATMENT

Cytotoxic agents

Anaplastic thyroid carcinoma cannot be regarded as a very chemosensitive tumor. Doxorubicin is not able to achieve more than a 20% response rate (Pacini et al., 1984). In a randomized study (Shimaoka et al., 1985) observed that combination chemotherapy based on doxorubicin (60 mg/m²) and cisplatin (40 mg/m²) was more effective than doxorubicin alone and provided a higher complete response rate. More recently, single drug docetaxel was tested as first-line chemotherapy in patients with advanced ATC. In a prospective phase II clinical trial of paclitaxel, 20 patients with metastatic ATC were enrolled and a remarkable response rate of 53% was obtained (Schoenberger et al., 2004). In a preclinical experiment (Schoenberger et al., 2004) only paclitaxel, gemcitabine, and vinorelbine appeared to be active in ATC (Bauer et al., 2003) and the combinations of vinorelbine/gemcitabine and paclitaxel/gemcitabine seemed to act synergistically. These results should receive confirmation in clinical trials.

Anti-angiogenetic agents

A common feature of thyroid cancers is their markedly increased vascularization, with an elevated expression of the vascular endothelial growth factor (VEGF) by immunohistochemistry, compared with normal thyroid tissue (Klein et al., 1999; Bauer et al., 2003). VEGF levels are correlated with stage, tumor size, nodal involvement, extra-thyroidal invasion, and distant metastases (Chaplin et al., 1996). On the basis of these findings, several drugs targeting angiogenesis have been evaluated against ATC.

- Combretastatin A4 phosphate (CA4P): is a tubulin-binding vascular disrupting agent that inhibits tumor blood flow. In contrast to other anti-angiogenetic drugs that block the formation of new vessels in tumors, vascular disrupting agents (such as CA4P) stop blood flow through already existing vessels, with the result of depriving tumor cells of oxygen and nutrients (McIver et al., 2001; Tozer et al., 2002). CA4P has activity against ATC cell lines and xenograft (Dowlati et al., 2002). In a phase I trial (Inai et al., 2004), one patient with ATC showed a progression-free survival of 30 months, however, the drug was found to be associated with significant cardiovascular side effects at the escalating doses employed.
- Axitinib (AG-013736) is an oral, potent, and selective inhibitor of VEGFRs 1, 2, and 3. Preclinical studies demonstrated that axitinib rapidly and selectively inhibits VEGF-dependent fenestrations and VEGFR-2 and 3 expression in endothelial cells, with the result of blocking angiogenesis and tumor blood flow (Baffert et al., 2006; Kamba et al., 2006; Mancuso et al., 2006; Mooney et al., 2009). The principal mechanism of action of axitinib is inhibition of VEGF signaling (Bauer et al., 2002).
- Bevacizumab (a monoclonal antibody anti VEGF) was tested alone and in combination with cetuximab in an *in vivo* model compared with doxorubicin. This study demonstrated that both drugs, either alone or in combination, inhibited tumor growth and angiogenesis better than doxorubicin (Prichard et al., 2007).
- AZD2171, a tyrosine-kinase inhibitor of the VEGFR-1 and VEGFR-2, blocked tumor growth and prolonged survival of ATC-bearing mice (Gomez-Rivera et al., 2007).

Histone deacetylase inhibitors

Histone deacetylase inhibitors are a promising class of antineoplastic agents that are able to induce cell differentiation, cell cycle arrest, and apoptosis through hyperacetylation of histones, with the potential to enhance the cytotoxicity of drugs such as doxorubicin. Preclinical studies have shown that valproic acid, a potent anti-convulsant agent, is able to enhance the activity of doxorubicin in cell lines derived from ATC alone or in combination with other drugs (Catalano et al., 2006; Kim et al., 2009).

Tyrosine-kinase inhibitors

- Imatinib (ST1571) is an oral inhibitor of the ABL kinase (the product of the fusion of Bcr and Abl gene). In addition, it can specifically inhibit c-Kit and PDGF receptors, which are hyper-functioning in some malignancies. On the basis of the assumption that ATC which overexpresses PDGFR and/or Abl might respond to imatinib.

- Sorafenib (Bay43-9006, Nexavar) is an oral, small tyrosine-kinase inhibitor of the Raf protein kinase receptor, VEGFR-2, and PDGF- β and displays strong anti-angiogenic activity. Sorafenib demonstrates an acceptable response rate in pre-treated ATC patients and further clinical studies are warranted.

Anti-EGFR agents

The epidermal growth factor receptor (EGFR) has been implicated in the pathogenesis of several types of cancer. There is supporting evidence that EGFR is expressed at high levels in ATC and papillary thyroid cancers (van der Laan et al., 1995). EGFR was expressed in all of the ATC cell lines examined and non-ligand dependent phosphorylation of EGFR was identified in half of the cell lines (Bergström et al., 2000). High expression of EGFR appears to be a negative prognostic factor in many types of tumors, but few studies have examined its prognostic role in thyroid cancers (Mizukami et al., 1991). Strong EGFR staining in papillary thyroid cancer was associated with poor prognosis (Akslen et al., 1993). These findings suggest that inhibition of EGFR may have anti-cancer efficacy in ATC.

- Gefitinib (ZD1839) is an orally active EGFR inhibitor that blocks EGFR-mediated downstream signal transduction. Pre-clinical trials have tested the activity of this drug against *in vitro* or *in vivo* models of ATC. Moreover Pennell et al studied the efficacy of gefitinib in a large group of thyroid cancer, including anaplastic thyroid cancer. Although gefitinib therapy did not result in any complete responses, the 32% of all patients underwent therapy with gefitinib have had reductions in tumor volume and prolonged stable disease, for the authors this may indicate biologic activity (Pennel et al., 2008).
- Cetuximab (C225) is a human-murine chimeric monoclonal antibody against EGFR. It has been approved by the Food and Drug Administration (FDA) for use in metastatic colorectal cancer and head and neck squamous cell carcinoma either metastatic or unresectable.

Agents targeting the NF- κ B pathway

The 26s proteasome is a large ATP-dependent multimeric complex that degrades intracellular proteins that have been marked for proteolysis by the process of ubiquitination (Adams, 2004). The ubiquitin-proteasome pathway plays a significant role in neoplastic growth and metastatic spread. The proteasome is also required for activating nuclear factor κ B (NF- κ B) by degradation of its inhibitory protein factor κ B inhibitor (I- κ B). NF- κ B is a transcription factor that upregulates a number of proteins involved in cancer progression including several anti-angiogenic and anti-apoptotic factors (Aghajanian et al., 2005).

- Bortezomib (PS-341) is a proteasome inhibitor that has been approved by the FDA for the treatment of multiple myeloma and its mechanisms of action include the inhibition of I- κ B, which leads to inactivation of the transcriptional factor NF- κ B (Davis et al., 2004; Papandreou et al., 2004). NF- κ B is often constitutively activated in medullary thyroid carcinoma and ATC, and is therefore implicated in their pathophysiology (Pacifico et al., 2004). Bortezomib has also been shown

to increase the expression of TRAIL (TNF-related-apoptosis-induced-ligand) receptors (TRAIL-R1 and 2) and to sensitize tumors to TRAIL-mediated killing (Conticello et al., 2007). The high cytotoxic activity and good *in vivo* tolerability of bortezomib holds promise for its future use in the treatment of ATC patients.

Agents targeting farnesyl-transferase

A new group of therapeutic agents called farnesyl-transferase inhibitors (FTIs) has been used in the treatment of solid tumors. Activating Ras mutations are common in thyroid cancers (Wynford-Thomas, 1997). Ras, the protein product of the Ras proto-oncogene, requires post-translational modification by conjugation of a farnesyl moiety to its C-terminal amino-acid. After farnesylation, Ras is localized to the inner surface of the cell membrane and is able to transduce the mitogenic signals mediated by tyrosine-kinase receptors. Farnesylation-blocking agents therefore operate by inhibiting Ras activity.

- Manumycin A is a natural product of *Streptomyces parvulus* that inhibits farnesyl-transferase and has antitumor activity against a variety of cancers *in vitro* and in xenograft models (Ito et al., 1996; Nagase et al., 1996). Apart from inhibition of angiogenesis, manumycin A causes apoptosis by inducing the pro-apoptotic protein Bax (Pan et al., 2005). No clinical trials have been performed to determine the activity and/or efficacy of manumycin A against ATC.

Proteasome inhibitor

PI3 kinase and MAPK pathways bind to heat shock protein 90 (HSP90). Disruption of HSP90 lead to reduced cell signaling and to cell death. Many cancer's cells demonstrated an over-activation of HSP90, so HSP90 inhibitors have been developed (Braga-Basaria et al., 2004).

- Geldanamycin is a benzoquinoid ansamycin antibiotic that works via the dissociation of the HSP90, affecting the stability and the steady state level of these oncoproteins. Park et al. (2003) demonstrated *in vitro* that geldanamycin inhibits thyroid cancer cell proliferation, down-regulates oncoproteins, and inhibits EGF-induced invasion. However Geldanamycin is weighed by important hepatic toxicity (Neckers, 2002).
- 17-Allylamino-17-demethoxygeldanamycin (17-AAG) 17-AAG maintains similar antitumor properties of geldanamycin but has fewer associated side effects. 17-AAG binds the ATP-binding pocket in the amino terminus of Hsp90, thereby inhibiting Hsp90 function (Neckers, 2002). Braga-Basaria and colleagues evaluated *in vitro* the activity of 17-AAG on cancer cells. Of the various cancer's cells lines, the most enhanced pro-apoptotic effects was demonstrated for those cell lines with highest level of HSP 90; thus, although not clearly applied yet to anaplastic thyroid cancer cells, the data suggest that HSP90 levels may serve as a biomarker for 17-AAG activity (Braga-Basaria et al., 2004).

Agents targeting matrix metalloproteinases

Matrix metalloproteinases (MMPs) are an important group of enzymes mediating the endothelial cell invasion and migration

required for the formation of new capillaries, a crucial step in the angiogenesis process.

- Minocycline is a semi-synthetic analog of tetracycline active against MMPs through chelation of the zinc ion at the active site of the enzyme. In a preclinical study, She and Jim (2006) investigated the effect of adding minocycline to manumycin A and paclitaxel against human ATC cells xenografted in nude mice, and demonstrated that the triple-drug combination resulted in the lowest average tumor growth rate, yielding significantly better survival than manumycin A alone, paclitaxel alone, or manumycin A plus paclitaxel. This novel combination deserves further investigation for the treatment of ATC.

Agents targeting PPAR γ

Peroxisome proliferator-activated receptor gamma (PPAR γ) agonists have demonstrated antitumor activity against a variety of human cancers in preclinical models and clinical trial (Zang et al., 2003). The mechanism of action of the different classes of these compounds, which comprise non-steroidal anti-inflammatory drugs, amino-acid derivatives, polyunsaturated fatty acids, eicosanoids, and thiazolidinediones, is attributed to the capacity of binding and activating PPAR γ . PPAR γ acts as a tumor suppressor gene, upregulating important enzymes which control the cell cycle (Nakajima et al., 2001).

Thiazolidinediones represent the most widely investigated pharmaceutical class among PPAR γ agonists (Aiello et al., 2006). In a preclinical study, two agents belonging to this class, ciglitazone and rosiglitazone, showed promising biological effects in ATC cells, such as an increased rate of apoptosis and inhibition of anchorage-dependent and -independent growth and migration. Furthermore, rosiglitazone increased the expression of thyroid-specific differentiation markers, thus inducing a partial reversion of the epithelial-mesenchymal transition in ATC cells, which correlates with ATC growth and dissemination (Weng et al., 2006).

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- RS5444 is another thiazolidinedione agent and a PPAR γ agonist. RS5444 demonstrated antitumor activity in preclinical studies, with a mechanism which includes the transactivation of genes regulating cell proliferation, apoptosis, and differentiation. In particular, PPAR γ activation is able to upregulate p21 protein, which is known to complex and inhibit an heterodimeric complex called cyclin dependent kinase 2 (CDK2)-cyclin E/A, responsible for cell cycle progression. Cells expressing nuclear p21 are subsequently arrested in the G0–G1 phase of the cell cycle (Pei and Xiong, 2005).

RADIATION

Radiation does not alter the course of ATC in most patients. On the other hand, when combined with surgery and chemotherapy, it can prolong the short-term survival in select and subset of patients. Intensity-modulated radiation therapy (IMRT) based on computerized treatment planning and delivery is able to generate a dose distribution that delivers radiation accurately with sparing of the surrounding normal tissue (Rosenbluth et al., 2005; Lee et al., 2007). Higher doses of radiation can be given over a shorter time with less toxicity by employing hyperfractionation techniques (Tennvall et al., 1990; Wong et al., 1991). Toxicity can be a limiting factor with radiation. Kim and Leeper (1987) reported complications particularly, pharyngitis, esophagitis, and tracheitis in their series. Wong et al. (1991) also noted skin changes, esophageal toxicity, and radiation myelopathy. Daily doses of greater than 3Gy should be cautiously used as it can increase the incidence of myelopathy (Wong et al., 1991).

More encouraging are the results reported by the concurrent use of taxanes and radiation. After standard dose of 60Gy in 30 fractions along with docetaxel 100 mg every 3 weeks for six cycles, an improvement of disease with partial remission (33%) and complete response (64%) was observed in ATC patients (Trocch et al., 2010; Brierley, 2011).

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Thyroid hormone deiodinases and cancer

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Deiodinases constitute a group of thioredoxin fold-containing selenoenzymes that play an important function in thyroid hormone homeostasis and control of thyroid hormone action. There are three known deiodinases: D1 and D2 activate the pro-hormone thyroxine (T4) to T3, the most active form of thyroid hormone, while D3 inactivates thyroid hormone and terminates T3 action. A number of studies indicate that deiodinase expression is altered in several types of cancers, suggesting that (i) they may represent a useful cancer marker and/or (ii) could play a role in modulating cell proliferation – in different settings thyroid hormone modulates cell proliferation. For example, although D2 is minimally expressed in human and rodent skeletal muscle, its expression level in rhabdomyosarcoma (RMS)-13 cells is threefold to fourfold higher. In basal cell carcinoma (BCC) cells, sonic hedgehog (Shh)-induced cell proliferation is accompanied by induction of D3 and inactivation of D2. Interestingly a fivefold reduction in the growth of BCC in nude mice was observed if D3 expression was knocked down. A decrease in D1 activity has been described in renal clear cell carcinoma, primary liver cancer, lung cancer, and some pituitary tumors, while in breast cancer cells and tissue there is an increase in D1 activity. Furthermore D1 mRNA and activity were found to be decreased in papillary thyroid cancer while D1 and D2 activities were significantly higher in follicular thyroid cancer tissue, in follicular adenoma, and in anaplastic thyroid cancer. It is conceivable that understanding how deiodinase dysregulation in tumor cells affect thyroid hormone signaling and possibly interfere with tumor progression could lead to new antineoplastic approaches.

Keywords: thyroid, deiodinase, cancer

INTRODUCTION

Deiodinases constitute a group of thioredoxin fold-containing selenoenzymes that metabolize thyroid hormone via two distinct pathways, i.e., thyroid hormone activation through outer ring deiodination (ORD) or thyroid hormone inactivation through inner ring deiodination (IRD; Bianco et al., 2002; Callebaut et al., 2003). D2 and D3 are expressed in multiple tissues, representing the main deiodinases involved respectively in activation and inactivation of thyroid hormone. In contrast, D1 expression is mostly observed in liver and kidney where it catalyzes both ORD and IRD of conjugated thyroid hormone. Thus, it has been suggested that D1 is a scavenger enzyme that recycles iodine from the backbone of inactive iodothyronine en route to elimination via bile or urine (Galton et al., 2009). In addition, lower D1 activity levels are also detected in other tissues, including the thyroid gland itself (Pereira et al., 2011). However, given its very low Km(T4) and Km(T3), it is questionable whether D1 in these other tissues plays a significant physiological role in euthyroid healthy individuals.

The expression of D2 and D3 can be exquisitely cell-specific and change rapidly in response to a number of developmental, metabolic, and disease cues through different signaling pathways (Gereben et al., 2008). Because the expression of these enzymes can be turned on or off in discrete groups of cells, most of the time their actions do not affect circulating thyroid hormone levels, which are tightly controlled via the TRH/TSH axis. Thus, the actions of D2 and D3 are viewed as a cell-specific pre-receptor

mechanism to control thyroid hormone signaling that cannot be predicted based on the levels of circulating thyroid hormone (Gereben et al., 2008). For example, stimulation of D2 expression in brown adipose tissue by the cAMP pathway accelerates transcription of T3-responsive genes such as UCP-1 and PGC-1, without elevating serum T3 levels (Hall et al., 2010). In fact, D2 has been shown to play a role in a number of systems by locally amplifying thyroid hormone action, e.g., interplay between astrocytes and neurons (Freitas et al., 2010), hypothalamus and the reproductive system (Yoshimura et al., 2003), and skeletal muscle (Dentice et al., 2011). In turn, ectopic D3 expression in the heart and brain during ischemia or hypoxia lifts the T3-dependent transcriptional footprint in these organs, in what can be seen as an adaptive mechanism to the disease state (Wassen et al., 2002; Olivares et al., 2007; Simonides et al., 2008; Pol et al., 2011).

Notably, a growing number of studies indicate that deiodinase expression is also altered in cancer (previously reviewed in Meyer et al., 2007; Piekielko-Witkowska and Nauman, 2011). While it is conceivable that deiodinase expression is unrelated to the cancer process, there is good indication that deiodinase reactivation could in some cases constitute a useful marker of the disease (Gereben and Bianco, 2009; Piekielko-Witkowska et al., 2009). In addition, it is also likely that the changes in thyroid hormone signaling resulting from deiodinase expression could play a role in cell proliferation and/or cell viability via affecting the expression of cycling D1, a protein factor that is part of a larger complex that controls

G1-S transition in the mitotic cell cycle (Dentice et al., 2007). This is illustrated in studies of basal cell carcinoma (BCC) cells, in which sonic hedgehog (Shh)-induced cell proliferation is accompanied by induction of D3 and loss of D2 activity. In fact, the growth of BCC cells implanted in mice is dramatically reduced after D3 expression is knocked down, indicating that dampening of thyroid hormone signaling is important for BCC growth (Dentice et al., 2007).

MODELS OF ALTERED DEIODINASE EXPRESSION IN CANCER CELLS

Given the multiple signaling pathways that regulate deiodinase expression, it is not surprising that in cells that normally have deiodinase activity the expression of these enzymes would be affected by the cancerous transformation (Table 1).

While D1 and D3 are transcriptionally regulated, D2 expression can be regulated both transcriptionally and post-translationally via ubiquitin-mediated D2 inactivation (Gereben et al., 2008). DIO1 is the human gene encoding D1, which consists of four exons and is located on chromosome 1 p32–p33 (Jakobs et al., 1997a). The gene is under the control of GC-rich SP1 promoters and contains two TREs in the 5' flanking region (FR), both contributing to its T3 responsiveness (Toyoda et al., 1995; Jakobs et al., 1997b; Zhang et al., 1998). In turn, the DIO2 gene is located on the long arm of the 14th human chromosome in position 14q24.3 (Celi et al., 1998; Araki et al., 1999). The coding region is divided into two exons by a ~7.4-kb intron (Celi et al., 1998).

It has three transcriptional start sites (TSS), 707, 31, and 24 bp 5' to the initiator ATG (Bartha et al., 2000). The human, mouse, and rat dio2 5'-FRs contain a functional cAMP responsive element (CRE; Bartha et al., 2000; Song et al., 2000; Gereben et al., 2001). In the human, dio2 5' FR functional, thyroid transcription factor-1 (TTF-1 or Nkx-2.1), Nkx-2.5, AP-1, and NF-κB sites have also been described (Gereben et al., 2001; Dentice et al., 2003; Zeold et al., 2006). In addition, glucocorticoids also increase D2 expression transcriptionally as established in GC pituitary tumor cells and in the chicken brain (Kim et al., 1998; Van der Geyten et al., 2001) via an actinomycin-dependent mechanism, not affecting the half-life of D2 mRNA. Lastly, in animals and in some cell models, LPS, and the NF-κB pathway have been shown to potently increase D2 mRNA levels and enzymatic activity, indicating that pro-inflammatory signals might also upregulate D2 expression (Fekete et al., 2005).

DIO3 is localized on the human chromosome 14q32 (Hernandez et al., 1998). In the mouse, the coding regions and the 3' UTR are contained in a single ~1.9 kb long exon. The D3 promoter contains a TATA box, two CAAT boxes, and several GC boxes in the proximal 180-bp region of the 5' FR (Hernandez et al., 1999). A conserved 180-bp-long enhancer was identified ~6 kb 3' to the dio3 TSS, and this region contains a consensus AP-1 site and serum response element (Hernandez and St Germain, 2003). A conserved Gli-2 (a member of the Gli transcription factor family that mediates Shh signaling) binding site, D3-A, is located in the mouse and human DIO3 5' FR (Dentice et al., 2007). Human and mouse DIO3

Table 1 | Summary of deiodinases changes in human malignancies.

Type of cancer	D1	D2	D3	Author/year
Follicular thyroid carcinoma	N/A	Increased	N/A	Kim et al. (2003)
Papillary thyroid carcinoma	Decreased	Decreased	N/A	Arnaldi et al. (2005)
Follicular thyroid carcinoma	Increased	Increased	N/A	
Papillary thyroid cancer	Decreased	Decreased	N/A	Ambroziak et al. (2005)
Papillary thyroid cancer	Decreased	N/A	N/A	de Souza Meyer et al. (2005)
Follicular thyroid carcinoma	Increased	Increased	N/A	
Anaplastic thyroid cancer	Increased	Increased	N/A	
Medullary thyroid cancer	N/A	Increased	N/A	Meyer et al. (2008)
Breast cancer	Increased	N/A	N/A	Debski et al. (2007)
Clear cell renal cell carcinoma	Decreased	N/A	N/A	Pachucki et al. (2001), Piekielko-Witkowska et al. (2009), Boguslawska et al. (2011)
Liver hemangioma	N/A	N/A	Increased	Huang et al. (2000), Peters et al. (2010), Balazs et al. (2007)
Lung cancer	Decreased	Unchanged	N/A	Wawrzynska et al. (2003)
Hepatic adenoma	Decreased	N/A	N/A	Sabatino et al. (2000)
Gliomas	N/A	N/A	Increased	Mori et al. (1993)
Astrocytoma	N/A	Decreased	N/A	Murakami et al. (2000)
Glioblastoma	N/A	Decreased	N/A	
Oligodendrogloma	N/A	Increased	N/A	
Astrocytoma	N/A	Increased	Decreased	Nauman et al. (2004)
Gliosarcoma	N/A	Increased	Increased	
Glioblastoma multiforme	N/A	Increased	Increased	
Prostate cancer	Decreased	N/A	N/A	Dutkiewicz et al. (1995)
Pituitary tumor	N/A	Increased	N/A	Baur et al. (2002)
Pituitary tumors	N/A	Increased	Increased	Tannahill et al. (2002)
TSH and ACTH producing tumors	N/A	Decreased	Increased	

genes map to chromosomal regions known to include imprinted genes and there is consensus that the DIO3 gene is imprinted, with preferential expression from the paternal chromosome (Hernandez, 2005). The expression of the DIO3 gene is regulated *in vivo* and *in vitro* by a number of different signaling pathways. Thyroid hormone up-regulates D3 activity in the rat brain (Peeters et al., 2001). In addition, D3 expression is also up-regulated by the action of serum, phorbol esters, and the epidermal and fibroblast growth factors (EGF, FGF; Hernandez and Obregon, 1995; Pallud et al., 1999; Hernandez and St Germain, 2002). High D3 activity can be found in human infant hemangiomas, a tumor enriched in blood vessels (Huang et al., 2000), indicating a relationship between D3 expression and angiogenic processes possibly through the basic FGF (bFGF) signaling pathway. In glial cells, induction of the DIO3 gene by growth factors appears to be mediated by the extracellular signal regulated kinases (ERKs; Pallud et al., 1999). In particular, D3 expression in astroglial cells is regulated by mitogens, growth factors, and hormones, and exposure to certain combinations of these agents results in synergistic induction of D3 mRNA levels and activity. The compounds that generate signals from the cell surface [tetradecanoyl phorbol acetate (TPA) and bFGF] induce rapid increases in D3 mRNA and activity, whereas treatment with ligands that interact with nuclear receptors (T3 and retinoic acid) result in slower effects. TGF- β stimulates transcription of the human DIO3 gene via a Smad-dependent pathway. Combinations of Smad2 or 3 with Smad4 stimulate the human DIO3 gene transcription only in cells that express endogenous D3 activity, indicating that Smads are necessary but not sufficient for D3 induction (Huang et al., 2005). TGF- β induces endogenous D3 in diverse human cell types, including fetal and adult fibroblasts from several tissues, hemangioma cells, and fetal epithelia. At the same time, D3 promoter activity is induced threefold to fourfold by estradiol, a mechanism that could contribute to the increased T4 requirements during human pregnancy (Alexander et al., 2003; Huang et al., 2003). During embryonic development, secondary epithelia trans-differentiates into mature epithelia or, under the influence of TGF- β and other paracrine factors, undergoes epithelial-mesenchymal transition to produce the various cell types of connective tissue. Thus, D3 expression in fetal epithelia can be retained through the process of epithelial-mesenchymal transition or reactivated after terminal differentiation by the action of TGF- β (Huang et al., 2005).

TYPE 1 DEIODINASE

D1 activity is readily detectable in the liver, kidney, and thyroid gland (Larsen et al., 1981). Notably, D1 expression is often suppressed in cancer cells compared with the healthy tissue. This is the case for example in the renal clear cell carcinoma (RCCC) where both D1 expression and activity were found to be undetectable compared with normal kidney cells (Pachucki et al., 2001). Furthermore D1 activity was studies in 44 patients with lung cancer (23 squamous cell and 21 adenocarcinoma) and found to be significantly lower as compared to peripheral lung tissue (Wawrzynska et al., 2003). Additionally, D1 activity is decreased in hepatic adenocarcinoma (Sabatino et al., 2000) and in prostate cancer tissue (Dutkiewicz et al., 1995).

Interestingly, an opposite pattern has been detected in different histological types of mammary gland tumors induced in Sprague-Dawley rats by injections of 1-methyl-1-nitrosourea (MNU). D1 activity was twofold higher in malignant mammary gland tumors compared with non-lactating mammary gland (Macejova et al., 2001). D1-mediated ORD was also tested in two breast cancer cell lines: MCF-7 (ovarian hormone-dependent) and MDA-MB-231 (ovarian hormone-independent). While D1 activity was present in MCF-7, which was stimulated only by retinoic acid treatments but not by T3 or the beta-adrenergic agonist isoproterenol, in MDA-MB-231 cells, no deiodinase activity could be detected in control conditions or under any of these treatments. These results suggest that D1 expression could represent a sensitive differentiation marker of breast cancer cells (Garcia-Solis and Aceves, 2003). More recently, the D1 activity was evaluated in 36 samples of breast cancers (grade G1 to G3) and in non-cancerous breast tissue taken from the opposite side to the location of the tumor. D1 activity in non-cancerous breast tissues was found to be very low or non-measurable. In contrast, in cancer tissues from the same breasts – especially in G1 and G2 tumors – D1 activity was significantly increased (Debski et al., 2007).

There are several studies assessing D1 expression in thyroid cancer. D1 mRNA and enzyme activity were noted to be significantly decreased in papillary thyroid cancer compared with the normal thyroid tissue, regardless of the histological subtype and/or the clinical stage (de Souza Meyer et al., 2005). Additionally, D1 gene expression was significantly lower in papillary thyroid cancer as assessed from a cDNA analysis of three thyroid carcinoma cell lines using 1807 open reading frame expressed sequence tags (ORESTES) previously recognized as cancer related genes (Arnaldi et al., 2005). In one study, even though both D1 activity and mRNA levels were found to be decreased in papillary thyroid cancer compared with healthy thyroid tissue, there was no correlation between protein expression and enzymatic activity, possibly due to both transcriptional and posttranslational mechanisms; it was also observed that there was a statistically significant correlation between D1 and Pax-8 expression in papillary thyroid tissue (Ambroziak et al., 2005).

In contrast, D1 expression was found to be higher in follicular thyroid cancer tissue and in follicular adenoma (de Souza Meyer et al., 2005). However, in follicular thyroid cancer cell lines D1 activity appeared to be present and to have a normal response to retinoic acid but lost the physiologic responsiveness to TSH and T3 (Schreck et al., 1994). D1 activity was evaluated in anaplastic cell carcinoma cell line as well and found to be undetectable, even after retinoic acid stimulation (Schreck et al., 1994); the opposite finding was obtained in other study (de Souza Meyer et al., 2005), with D1 activity significantly higher in one sample of anaplastic cancer compared with normal tissue. This opposite result, if confirmed, may be due to the different histological characteristic present before the dedifferentiation process took place.

TYPE 1 DEIODINASE EXPRESSION AS A MARKER OF HUMAN RENAL CANCER

As mentioned above, D1 is highly expressed in the normal kidney. However, DIO1 expression is reduced in the most common subtype of renal cancer, i.e., the RCCC. Both D1 expression and

activity were undetectable in RCCC tissues (Pachucki et al., 2001). Additionally, there seems to be a loss of the normal correlation between D1 mRNA and activity in these tissues, possibly as a result of post-transcriptional regulation. Studies from the same group expanded their initial findings reporting interesting results related to alternative splicing of DIO1 (Piekielko-Witkowska et al., 2009). They cloned and identified a total of 11 D1 mRNA transcripts, seven of which were previously unreported. Of the 11 variants, all were expressed in the RCCC samples, even if in significantly lower amount compared with the control groups, while only eight were present in healthy renal tissue. These findings lead to the conclusion that three new splicing variants were expressed exclusively in the cancer cells suggesting that they could potentially be used as unique molecular markers for kidney tumors. All the putative D1 protein encoded by these three new variants are truncated products of 111, 115, and 14 amino acids. In the last two alternative splice forms the premature termination codon (PTC) is located more than 50 nucleotides upstream of the final exon–exon junction and usually they are degraded by the nonsense-mediated mRNA decay (NMD) mechanism. Additionally all of them would be inactive since they lack the exon 2 region that encodes the enzyme's active center. These observations could explain the original finding of undetectable D1 expression and activity in RCCC tissue.

Recently the same authors investigate microRNAs (miRNAs) as alternative regulators of DIO1 expression and function (Boguslawska et al., 2011). MicroRNAs bind to complementary sequences of target mRNAs and behave as post-transcriptional regulators interfering with translation or causing target degradation. Using bioinformatic analysis they identify seven potential miRNA targeting regions of the 3' untranslated region (3' UTR) of DIO1 mRNA, two of which (miR-224 and miR-383) were significantly over expressed in RCCC compared with normal tissue. They also observed that there was a significant reduction of DIO1 transcript in the clear cell carcinoma cell line Caki-2 which was previously transfected with miR-224 precursor. Additionally the introduction of anti-miR-224 in these cells increased DIO1 expression by 45%. Furthermore in miR-224 and miR-383 transfected Hela cells a decrease of the activity of a luciferase reporter containing the 3' UTR of DIO1 was observed. This decrease was abolished when mutated constructs were used instead, suggesting that these miRNAs directly bind to DIO1 3' UTR. Finally miR-224 expression in RCCC cells was found to correlate negatively with DIO1 mRNA content and T3 concentration suggesting that miR-224 induce intracellular hypothyroidism via a loss of DIO1 function. Taken together these results open the possibility of an important regulatory role of microRNAs in deiodinase activity particularly in cancer cells.

TYPE 2 DEIODINASE

D2 is highly expressed in the brain with its mRNA and activity normally present in astrocytes and other glial cells where it participates in the paracrine control of T3-responsive genes in neurons (Freitas et al., 2010). However, D2 expression is much higher in most brain tumors such as astrocytoma and glioblastoma with the highest D2 activity in gliosarcomas and oligodendrogiomas (Mori et al., 1993; Murakami et al., 2000; Nauman et al., 2004). Remarkably, even neuroblastomas express D2, given that normal

neurons are not known for exhibiting D2 activity (St Germain, 1986). Still within the central nervous system, the pituitary gland is another structure that normally expresses D2, specifically in the TSH-producing cells, participating in the normal TSH feedback mechanism (Christoffolete et al., 2006). D2 mRNA levels in 105 pituitary tumors were found to be 2.6-fold increased in all pituitary tumors with the highest expression observed in non-functional adenoma when compared with normal pituitary tissue. The only exceptions were the TSH and ACTH producing tumors where D2 mRNA was actually reduced (0.1-fold; Tannahill et al., 2002). A higher D2 activity in TSH- and PRL-producing adenomas was also reported, with variable D1/D2 ratios among patients with similar types of tumors (Baur et al., 2002).

Several neoplastic cell lines were found to exhibit high D2 expression as compared with their normal counterparts. For example, D2 is usually expressed in placenta and is also present in JEG3, a choriocarcinoma cell line (Canettieri et al., 2000). In these cells, D2 has been shown to be highly responsive to cAMP treatment that involves the binding of transcription factor CRE binding protein (CREB) to the CRE located in the hD2 promoter (Canettieri et al., 2000). Similar levels of D2 activity were reported in normal lung tissue as well as in lung cancers (squamous cell cancer and adenocarcinoma; Wawrzynska et al., 2003). At the same time, D2, which is expressed in mesothelial cells, has 40-fold higher expression in the mesothelioma cell line (MSTO-211H), with the highest levels of D2 ever seen in cultured cells (Curcio et al., 2001). Furthermore, D2 is only minimally expressed in human and rodent skeletal muscle or in primary cultures (Grozovsky et al., 2009; Ramadan et al., 2011), however its expression is much higher in rhabdomyosarcoma (RMS)-13 cells (da-Silva et al., 2007).

The expression of D2 mRNA and the presence of D2 activity were detected in human osteoblast-like osteosarcoma (SaOS-2) cell line but this time in lower amount compared with the normal human osteoblast (NHOst) cells (Gouveia et al., 2005). Interestingly, TSH was able to increase equally D2 mRNA expression and activity in both cell lines via a TSH receptor-cAMP mediated pathway suggesting that transcriptional regulation of D2 may play an important role in the homeostasis of human osteoblasts (Morimura et al., 2005).

D2 is also expressed in normal human thyroid tissue but its expression changes in thyroid adenomas and cancer. D2 mRNA and activity was found to be significantly increased in hyperfunctioning thyroid adenoma compared with the normal tissue (Murakami et al., 2001). In follicular carcinoma, D2 has increased activity as well. In three patients with large or widely metastatic follicular thyroid carcinoma, there was a persistent increment of the ratio of serum T3 to T4 in the absence of autonomous production of T3 by the tumor. D2 activity was analyzed in one of these patient and was found to be eightfold up-regulated compared with the normal tissue. Resection of the tumor normalized the serum T3 to T4 ratio (Kim et al., 2003). Similarly in anaplastic thyroid cancer D2 activity was found to be higher than normal thyroid tissue (de Souza Meyer et al., 2005). In contrast D2 mRNA and activity are decreased in papillary thyroid cancer compared with the normal thyroid cells (Arnaldi et al., 2005; de Souza Meyer et al., 2005). In one study they observed poor correlation between the low D2 mRNA level and the enzymatic activity in papillary

thyroid cancer but a statistically significant correlation between D2, Pax-8, and Titf1/Nkx-2 mRNA expression suggesting a potential role in the impairment of deiodinase expression (Ambroziak et al., 2005). D2 is also highly expressed in human medullary thyroid carcinoma (MTC), with activity that was comparable to those found in surrounding normal follicular tissue (Meyer et al., 2008).

TYPE 3 DEIODINASE

Type 3 iodothyronine deiodinase (D3), the main physiological inactivation mechanism of thyroid hormone, is widely expressed during embryonic life. However, after birth D3 expression subsides in most tissues while remaining present in the human placenta, endometrium, skin, and brain of healthy adults (Bates et al., 1999; Huang, 2005). Interestingly, D3 activity can be reactivated in many tissues in disease states by signals such as hypoxia or ischemia, as well as in tumoral tissues (Huang and Bianco, 2008). For example, D3 activity was evaluated in different brain tumors and compared with normal tissue and found to be increased in all the eight cases of gliosarcoma and in 9 out of 10 cases of glioblastoma multiforme. Additionally the concentration of T3 and T4 were significantly lower in glioma than in the non-tumoral brain tissue samples, indicating that D3 expression was decreasing thyroid hormone signaling locally. On the contrary, the activity of D3 was found to be decreased in all cases of astrocytoma regardless of their grade (Nauman et al., 2004).

D3 expression was also evaluated in 105 pituitary tumors and 10 normal pituitaries. In the tumors, there was significant increase in D3 mRNA compared with the normal tissue, especially in the tumors producing TSH (13-fold), ACTH (sevenfold), GH (six-fold), and the non-functional ones (sevenfold). However, despite the increase in D3 mRNA, D3 enzymatic activity was increased in only three non-functional tumors of the 16 analyzed in total (Tannahill et al., 2002).

D3 expression was evaluated as well in several neoplastic cell lines. D3 mRNA was detected in endometrium carcinoma (ECC-1), mamma carcinoma (MCF-7), and neuroblastoma (SH-SY5Y), but not in the hepatocarcinoma (HepG2), choriocarcinoma, or astrocytoma cell line. Phorbol ester 12-O-tetradecanoylphorbol-13-acetate, a tumor promoter, increased D3 activity twofold to ninefold in ECC-1, MCF-7, and SH-SY5Y cells. In turn, estradiol increased D3 activity threefold only in ECC-1, suggesting its potential role in regulating D3 expression in endometrium during pregnancy. Incubation with retinoids increased D3 activity twofold to threefold in ECC-1 and MCF-7 cells but decreased D3 activity in SH-SY5Y cells. Finally, they also observed in all the cell lines a loss of D3 response to known physiologic stimuli such as T3, possibly due to the underling neoplastic process (Kester et al., 2006).

High levels of D3 activity have been reported in vascular benign tumors like infantile hemangiomas and hepatic hemangiopericytoma (Huang et al., 2000; Ruppe et al., 2005). In many cases the D3 activity level is so high that may result in thyroid function abnormalities due to the accelerated rate of thyroid hormone degradation. This results in subclinical hypothyroidism and even in clinically relevant hypothyroidism (Murakami et al., 2001). The first patient with consumptive hypothyroidism described was a 3-month-old infant with hepatic hemangiomas

and severe hypothyroidism refractory to the standard dose of thyroid hormones replacement (Huang et al., 2000). Such patients may improve with T3 replacement treatment (Peters et al., 2010) or by the surgical removal of the tumor as suggested by a case of a patient with consumptive hypothyroidism and liver hemangiopericytoma cured by liver transplantation (Balazs et al., 2007).

Recently, a cluster of 23 up-regulated miRNAs was identified in mice liver tumors and encoded within the Dlk1-Gtl2 imprinted locus on chromosome 12qF1. This region maps to the human DLK1-DIO3 region on chromosome 14q32.2. The expression of DLK1-DIO3 miRNA was examined in 97 patients with hepatocellular carcinoma (HCC) associated with hepatitis B infection. Eighteen of such patients exhibited a strong overexpression of miRNAs which was not observed in other previously tested cancers such as breast, lung, kidney, stomach, or colon. Furthermore, the increased expression of the DLK1-DIO3 miRNA was found to be correlated with some HCC stem cell markers, with a high level of serum α -fetoprotein and a poor survival rate suggesting that DLK1-DIO3 miRNA may be used as a marker for those subtypes of HCC associated with poor prognosis (Luk et al., 2011).

WHAT IS THE RESULT OF DEIODINASE-MEDIATED CHANGES IN THYROID HORMONE SIGNALING IN CANCER?

Given that in some settings (e.g., BCC) thyroid hormone reduces cell proliferation (Dentice et al., 2007), could a deiodinase dysregulation in tumor cells affect thyroid hormone signaling and thus interfere with tumor progression? This question stems from the modern paradigm that thyroid hormone signaling can be regulated relatively independent of plasma thyroid hormone levels, in a time- and tissue-specific fashion by the deiodinases (Gereben et al., 2008). In fact, in the developing chicken growth plate, loss of D2 activity via Shh-induced D2 ubiquitination has been linked to parathyroid hormone-related peptide (PTHrP) expression and chondrocyte proliferation (Dentice et al., 2005).

These studies prompted investigators to look into other settings in which the Shh signaling pathway is active, such as the BCC, the most common human malignancy characterized by a constitutively active Shh pathway (Dentice et al., 2007). In these cells, Shh increases D3 expression via a Gli-2-mediated transcriptional mechanism, which reduces intracellular T3 concentrations. This effect synergizes with the Shh-stimulated ubiquitin inactivation of D2, which further decreases the intracellular levels of T3. The decrease in thyroid hormone signaling results in an increase in cyclin D1, increasing cell proliferation. Subsequently, a specific D3 shRNAi construct (iD3) was transfected into BCC cells and thymidine uptake experiments showed that D3 depletion significantly reduced cyclin D1 levels and therefore proliferation. Furthermore, a rescue experiment by reintroducing a functional human D3 gene in D3-depleted cells resulted once again in increased cyclin D1 levels and cell proliferation, confirming that D3 plays a key role in cell cycle in these cells. Interestingly, a fivefold reduction in the growth of BCC in nude mice was observed if nude mice receiving BCC xenografts in which D3 expression was knocked down (Dentice et al., 2007). At this writing, it is unclear that such

a mechanism would operate in other tumors that also express deiodinases.

At the same time, an increase in thyroid hormone signaling accelerates the metabolic rate and the oxidation of energy substrates such as glucose and fatty acids in most cell types (Bianco et al., 2005). Cancer cells are known to have accelerated metabolism and increased glycolysis (Koppenol et al., 2011). Thus, it is conceivable that, by affecting thyroid hormone signaling, deiodinases could interfere with the metabolism of cancerous cells. If confirmed, this could also constitute a potential therapeutic strategy for certain types of cancers that depend on a high metabolic rate.

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CONCLUSION

Deiodinases are enzymes that can up- or down-regulate thyroid hormone signaling on a cell-specific basis, independently of circulating thyroid hormone levels. Several types of cancers and cancerous cell lines express high (low) levels of deiodinases that could contribute to the loss in control of cell division and consequently tumor development; this could also potentially affect their metabolic rate and selection of oxidative substrates. Understanding the mechanisms underlying the dysregulation in deiodinase expression in tumor cells as well as the downstream impact of changes in thyroid hormone signaling could potentially lead to the development of new antineoplastic approaches.

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Epigenetics modifications and therapeutic prospects in human thyroid cancer

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At present no successful treatment is available for advanced thyroid cancer, which comprises poorly differentiated, anaplastic, and metastatic or recurrent differentiated thyroid cancer not responding to radioiodine. In the last few years, biologically targeted therapies for advanced thyroid carcinomas have been proposed on the basis of the recognition of key oncogenic mutations. Although the results of several phase II trials look promising, none of the patients treated had a complete response, and only a minority of them had a partial response, suggesting that the treatment is, at best, effective in stabilizing patients with progressive disease. "Epigenetic" refers to the study of heritable changes in gene expression that occur without any alteration in the primary DNA sequence. The epigenetic processes establish and maintain the global and local chromatin states that determine gene expression. Epigenetic abnormalities are present in almost all cancers and, together with genetic changes, drive tumor progression. Various genes involved in the control of cell proliferation and invasion (p16INK4A, RASSF1A, PTEN, Rap1GAP, TIMP3, DAPK, RAR β 2, E-cadherin, and CITED1) as well as genes specific of thyroid differentiation (Na $^{+}$ /I $^{-}$ symport, TSH receptor, pendrin, SL5A8, and TTF-1) present aberrant methylation in thyroid cancer. This review deals with the most frequent epigenetic alterations in thyroid cancer and focuses on epigenetic therapy, whose goal is to target the chromatin in rapidly dividing tumor cells and potentially restore normal cell functions. Experimental data and clinical trials, especially using deacetylase inhibitors and demethylating agents, are discussed.

Keywords: advanced thyroid cancer, anaplastic thyroid cancer, target therapy, epigenetic, histone deacetylase inhibitors, demethylating agents

INTRODUCTION

Thyroid carcinoma is the most common endocrine malignancy worldwide, its incidence being approximately 1–5% of all cancers in females and less than 2% in males. Even though it is considered a relatively rare neoplasm, its incidence is progressively increasing (Kilfoyle et al., 2009). Thyroid carcinomas arising from follicular epithelial cells (about 95% of all thyroid tumors) are traditionally classified as well-differentiated thyroid carcinomas (WDTC), including both papillary (PTC, 80%) and follicular types (FTC, 10–15%; Fassnacht et al., 2009), poorly differentiated thyroid carcinomas (PDTC; Patel and Shaha, 2006; Ghossein, 2009) and, finally, anaplastic thyroid carcinoma (ATC), accounting for 1–2% of thyroid malignancies. WDTC usually share slow growth rate and high percent of cure achieved by a combination of surgery, radioiodine ablation, and TSH-suppressive therapy. Poorly differentiated tumors, either from the beginning or loosing differentiation as disease progresses, as well as ATC, are generally resistant to conventional therapies, and no successful treatment is now available (Smith et al., 2009; Catalano et al., 2010; Sherman, 2011). Medullary thyroid carcinoma (MTC) represents a minority of thyroid cancers arising from para-follicular calcitonin producing cells. Neuroendocrine-derived MTC is not responsive to either radioiodine or TSH suppression and its treatment is mainly based on surgery for primary and regional metastatic disease. The

outcome of patients with metastatic disease is similar to that of radioiodine-unresponsive WDTC (Roman et al., 2006).

The term advanced thyroid cancer refers to all thyroid tumors resistant to conventional therapies, and comprises PDTC, ATC, and metastatic or recurrent differentiated cancers which do not respond to radioiodine. Chemotherapy is generally taken into consideration only for patients with symptomatic or rapidly progressive metastatic disease unresponsive to or unsuitable for surgery, radioiodine, and external beam radiotherapy; but the efficacy of cytotoxic systemic chemotherapies for these tumors is fairly poor, response rates being around 25% or less (Sherman, 2010).

In the recent past, biologically targeted therapies for advanced thyroid carcinomas have been proposed on the basis of the recognition of key oncogenic mutations, but also of adaptation processes facilitating tumor growth, such as hypoxia-inducible angiogenesis or epigenetic modifications of chromosomal DNA and histones.

As far as oncogenes are concerned, RAS mutation may be an early step in thyroid tumorigenesis, as it is present with frequencies ranging from 24 to 53% in follicular adenomas (FA) and from 18 to 52% in FTC (Nikiforova et al., 2003a). In FTC, PAX8-PPAR γ rearrangement was also reported (Kroll et al., 2000). RET/PTC rearrangement, distinctive of PTC, has not been identified in FA or FTC, supporting divergent tumor induction/progression

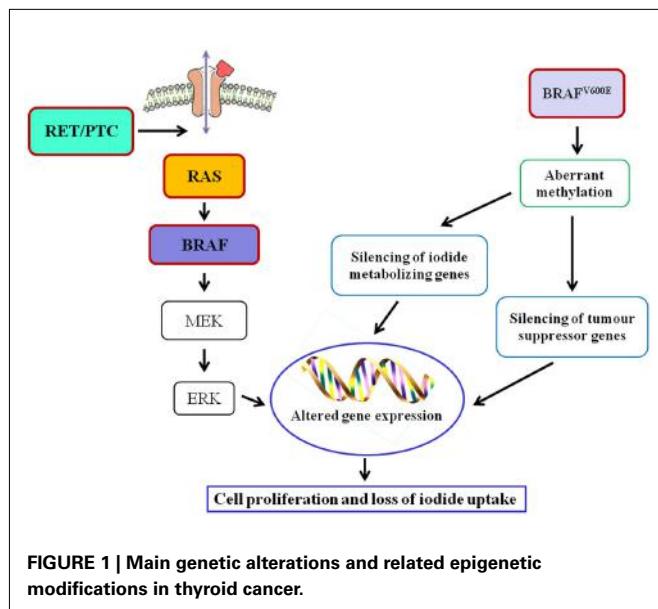


FIGURE 1 | Main genetic alterations and related epigenetic modifications in thyroid cancer.

(Wreesmann and Singh, 2008). The majority of PTC, in fact, are initiated by well characterized genetic events, involving single activating somatic mutation of BRAF or RAS, and translocations producing RET/PTC oncogenes (Fagin, 2004). BRAF^{V600E} mutation, found in approximately 40% of PTC (Ciampi and Nikiforov, 2005), leads to constitutively active BRAF and subsequent activation of the RET/RAS/BRAF/MAPK signal transduction pathway and, in addition, the presence of BRAF mutations is associated with decreased expression of mRNAs for the sodium iodide symporter (NIS) and the TSH receptor, that are considered markers of thyroid differentiation (Durante et al., 2007; **Figure 1**). Mutated BRAF is also associated with the PTC to PDTC progression, since it makes thyroid cells sensitive to TGF β -induced EMT (epithelial–mesenchymal transition; Knauf et al., 2011) and it is involved in the control of some ECM non-cellular components such as thrombospondin-1; it was reported that BRAF^{V600E} knock-down caused a reduction of TSP-1 expression and, as a consequence, of adhesion and migration/invasion of human thyroid cancer cells (Nucera et al., 2010, 2011). Moreover, treatment of mice carrying BRAF^{V600E} PTC with BRAF inhibitors reduced tumor proliferative index and partially restored thyroid-specific gene expression (Chakravarty et al., 2011). Finally, in PTC the pro-angiogenic factor vascular endothelial growth factor (VEGF) expression correlates with a higher risk of metastasis and recurrence, a shorter disease-free survival, and BRAF mutation status (Lennard et al., 2001; Jo et al., 2006).

Poorly differentiated thyroid carcinomas and ATC have a more complex and less distinct gene profile and molecular data suggest that these aggressive forms may dedifferentiate from WDTC. For instance, the well-known mutations of BRAF and RAS occurring in PTC are present in more than 50% of PDTC (Ghossein, 2009). In about one-third of anaplastic carcinomas, probably those arisen from pre-existing PDTC, is present the BRAF^{V600E} mutation (Nikiforova et al., 2003b). It was also suggested that ATC might develop from RAS-mutated FTC since RAS mutation was

also detected in 6–50% of ATC (Smallridge et al., 2009). Further, in ATC a mutation of PI3KCA gene causing Akt and ERK activation was reported (Smallridge et al., 2009). Additional mutations frequently observed in ATC involve p53 and β -catenin. The tumor-suppressor gene p53 is fundamental for the progression from indolent to aggressive thyroid cancer. The inactivating p53 mutation, seldom detected in WDTC, is found in about 55% of PDTC and ATC (Smallridge et al., 2009). Membrane β -catenin expression is progressively reduced with loss of tumor differentiation, resulting in tumor invasiveness, and increasing metastatic potential (Garcia-Rostan et al., 2001).

As far as MTC is concerned, heritable germ-line activating mutations in RET are found in almost all familial cases and identical somatic mutations in sporadic disease. Activated RET mutant proteins also enhance MAPK signaling (Santoro et al., 1995).

The use of selective inhibitors of activated BRAF, RET, and RET/PTC kinases as well as of VEGF and VEGF receptor to treat advanced thyroid cancer is under thoughtful evaluation. To date, a number of clinical trials regarding tyrosine kinase and angiogenic factors inhibitors are in progress^{1,2}.

EPIGENETIC ALTERATIONS

In the early 1940s, Conrad Hal Waddington coined the term epigenetics as “the causal interactions between genes and their products, which bring the phenotype into being” (Waddington, 1942). Currently, epigenetic refers to the study of heritable changes in gene expression that occur without any alteration in the primary DNA sequence (Sharma et al., 2010). The epigenetic processes establish and maintain the global and local condensed or decondensed chromatin states that determine gene expression. The continuous interplay of all these processes is today called “epigenome” – the epigenetic status that determines the way a single eukaryotic genome may manifest in different cell types and developmental stages and that, if aberrant, gives rise to cancer and other diseases. In fact, epigenetic abnormalities are present in almost all cancers and, together with genetic changes, drive tumor progression. Moreover, acting in concert with genetic changes, they play a role in the earliest steps of tumorigenesis (Feinberg et al., 2006), as also suggested by the growing list of tumor-suppressor genes that are often epigenetically silenced but rarely genetically mutated in the pre-invasive stages of many cancers (Jones and Baylin, 2007).

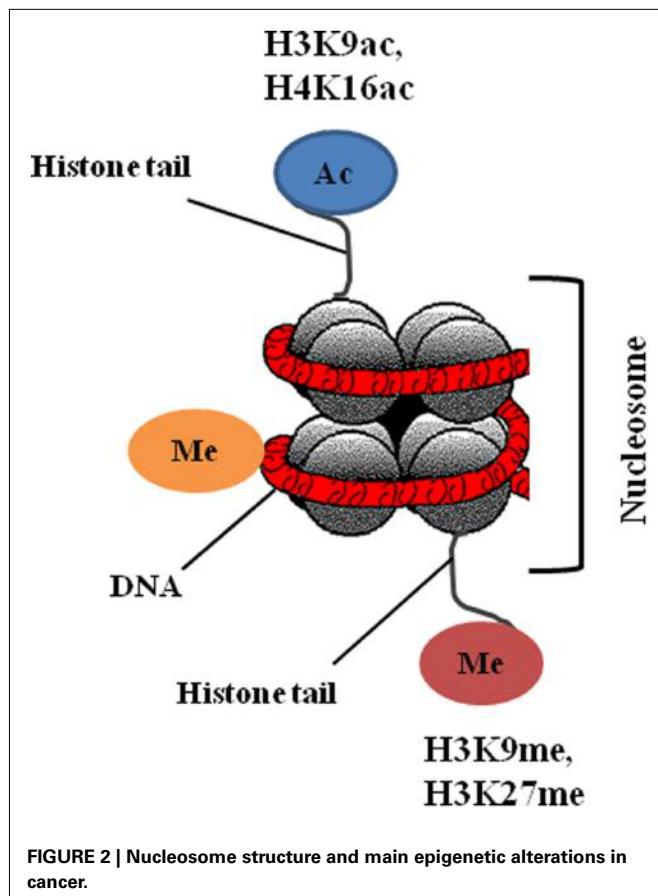
Epigenetic information that fulfills the requirement of heritability can be classified into three distinct types: DNA methylation, histone modifications, and non-coding RNAs.

In the present review, we will primarily discuss DNA methylation, and histone modifications (**Figure 2**), as drugs that target these epigenetic modifications are already at a clinical developmental stage.

DNA methylation takes place within the CpG dinucleotides, and its consequence is the silencing of genes and non-coding genomic regions. There are three main DNA methyltransferases (DNMTs): DNMT1, which maintains the existing methylation patterns following DNA replication, and DNMT3A and DNMT3B, which target previously unmethylated CpGs. Cancer genome

¹www.thyroid.org

²www.clinicaltrials.gov



is typically characterized by global hypomethylation concomitant with hypermethylation of CpG islands in the promoters of genes that play important roles in regulating cell cycle, apoptosis, differentiation, and cell adhesion (Baylin and Herman, 2000).

Post-translational modifications of the N-terminal tails of histones include acetylation, methylation, phosphorylation, ubiquitination, SUMOylation, and ADP ribosylation. Histone modifications can lead to either gene activation or repression, depending upon which residues are modified and the type of modification (Chi et al., 2010). Overall, histone modifications affect chromatin conformation and consequently influence gene transcription, DNA repair and replication, and cell cycle checkpoints (Sawan et al., 2008). Histone acetylation and deacetylation cause activation and arrest of gene transcription, respectively, and the enzymes that catalyze these changes, histone acetyltransferases (HATs) and histone deacetylases (HDACs), can also target non-histone proteins such as p53, Hsp90, and α -tubulin (Sterner and Berger, 2000). There are four classes of HDACs: class I consists of HDAC 1, 2, 3, and 8; class II consists of HDAC 4, 5, 6, 7, 9, and 10; class III are sirtuins (SIRT 1–7); and class IV consisting of HDAC 11. Alterations in histone modifications significantly contribute to the onset and progression of cancer (Chi et al., 2010). The most common epigenetic modifications of histones in cancer are acetylation and methylation; reduction in monoacetylated H4-K16 and trimethylation of H4-K20 are general features of cancer cells (Fraga et al., 2005). Altered expression of HDACs has been

Table 1 | Epigenetic modifications in thyroid cancer.

	Gene	Reference
Genes involved in control of proliferation and invasion	p16INK4A RASSF1A PTEN Rap1GAP TIMP3 DAPK RAR β 2 E-cadherin	Elisei et al. (1998) Schagdarsurengin et al. (2002) Alvarez-Nuñez et al. (2006) Zuo et al. (2010) Hoque et al. (2005), Hu et al. (2006)
Thyroid-specific genes	CITED1 Na $^{+}$ /I $^{-}$ symport TSH receptor pendrin SLC5A8 TTF-1	Graff et al. (1998), Wiseman et al. (2006) Sassa et al. (2011) Xing (2007a), Xing (2007b)
		Kondo et al. (2009)

reported in tumor samples; overexpression of class I HDACs has been reported in 5–40% of cancer tissues (Nakagawa et al., 2007), HDAC1 is overexpressed in prostate, gastric, colon, and breast carcinomas. HDAC2 is overexpressed in colorectal, cervical, and gastric cancer, whereas overexpression of HDAC6 is observed in breast cancer specimens (Ellis et al., 2009).

MicroRNAs (miRs), are small (19–25 nucleotides), non-coding RNA molecules representing the most recent class of molecules known to be involved in epigenetic regulation. They function as negative regulators of the expression of protein-encoding genes, involved in major processes such as development, apoptosis, cell proliferation, immune response, and hematopoiesis.

EPIGENETIC MODIFICATIONS IN THYROID CANCER

Various genes involved in the control of cell proliferation and invasion as well as genes specific of thyroid differentiation are epigenetically silenced in thyroid cancer (Table 1). Cumulative epigenetic alterations play a role in the sequential progression from indolent WDTC to metastasizing carcinomas, through the spectrum of poorly differentiated to undifferentiated thyroid carcinoma.

DNA METHYLATION

In thyroid cancer, aberrant methylation of tumor-suppressor genes is common. CDK inhibitors, such as p27KIP1 and p16INK4A, are commonly down-regulated; methylation of CpG islands in p16INK4A is detected in 30% of thyroid neoplasms (Elisei et al., 1998).

The tumor-suppressive Ras effector, RAS association domain family 1, splicing isoform A (RASSF1A) contains a Ras association domain, and plays a role in the regulation of cell cycle and apoptosis (Donninger et al., 2007). In the thyroid, RASSF1A promoter methylation is present in more than 30% of benign and malignant thyroid tumors. The high frequency of RASSF1A hypermethylation both in benign FA (33–44%) and the increased occurrence in FTC (70–100%) suggest that epigenetically silencing of RASSF1A

is an early step in thyroid tumorigenesis (Schagdarsurengin et al., 2002).

PTEN, a phosphatase that terminates the PI3K/Akt pathway, has been found aberrantly methylated in about 50% of papillary carcinomas and almost 100% of follicular carcinomas and adenomas, suggesting that it may be involved in thyroid tumorigenesis (Alvarez-Nuñez et al., 2006).

Rap1GAP is a Rap1 GTPase-activating protein that inhibits the RAS superfamily protein Rap1 by facilitating hydrolysis of GTP to GDP. In human thyroid cancers, Rap1GAP expression is frequently lost or down-regulated secondary to promoter hypermethylation and/or loss of heterozygosity; Rap1GAP loss of expression correlates with tumor invasiveness (Zuo et al., 2010).

A close association between *BRAF* mutation and aberrant methylation of several tumor-suppressor genes in PTC, including the genes for tissue inhibitor of matrix metalloproteinase-3 (TIMP3), death-associated protein kinase (DAPK), and retinoic acid receptor β 2 (RAR β 2) has been reported (Hoque et al., 2005). This association correlated with high-risk clinicopathological characteristics of PTC, including extra-thyroid invasion, lymph node metastasis, and advanced disease stages (III and IV; Hu et al., 2006). TIMP3 suppresses tumor growth, angiogenesis, invasion, and metastasis both by preventing the interstitial matrix destruction promoted by matrix metalloproteinase (MMP)-3 and by blocking the binding of VEGF to the VEGF receptor (Anand-Apte et al., 1996). Therefore, methylation-mediated silencing of the *TIMP3* gene may play a unique role in *BRAF* mutation-promoted invasiveness and progression of PTC.

As far as invasiveness is concerned, E-cadherin joins together with catenins to promote Ca^{2+} -dependent, homotypic cell-to-cell adhesion and to establish normal epithelial tissue architecture. Disruption of the E-cadherin/catenin complex contributes to tumor metastasis, and decreased expression of E-cadherin is observed in advanced stage, poorly differentiated carcinomas, (Graff et al., 1998) and is associated with the transformation of differentiated into ATC (Wiseman et al., 2006).

Aberrant methylation also involves thyroid-specific genes such as the Na^+/I^- symporter (NIS), the promoter of the TSH receptor, the genes for the putative thyroid follicular cell apical iodide transport (pendrin and SCL5A8; Xing 2007a,b). Suppression of these thyroid iodide-metabolizing molecules results in the loss of cancer cells ability to concentrate iodine, rendering tumors insensitive to radioiodide therapy.

Hypomethylation of the CpGs in the promoter region of CITED1 (Cbp/p300 Interacting Transactivators with glutamic acid [E] and aspartic acid [D]-rich C-terminal domain) is associated with higher expression of CITED1 mRNA in PTC tissues (Sassa et al., 2011).

HISTONE MODIFICATIONS

Unfortunately, little information about the histone modifications present in thyroid tumors and the relationship between such modifications and thyroid cancer behavior is at present available.

However, recently, Puppin et al. (2011) investigated whether global levels of acetylated histones are modified in thyroid cancer tissues. They show that levels of acetylated H3 at residue K18 are lower in undifferentiated cancers with respect to differentiated

Table 2 | Epigenetic drugs in clinical trials for the treatment of advanced thyroid cancer (www.clinicaltrials.gov and <https://oss-sper-clin.agenziafarmaco.it>).

Drug	Epigenetic target	Developmental stage
Decitabine	DNMT	Phase II
Depsipeptide	HDAC1,2	Phase I/II
Vorinostat (SAHA)	HDACs (class I, IIa, IIb, IV)	Phase II
Valproic acid (VPA)	HDACs (class I, II)	Phase II/III
Panobinostat (LBH589)	HDACs (class I, IIa, IIb, IV)	Phase II

tumors, suggesting that acetylation is switched off in the thyroid tumor transition.

CpG hypermethylation in the promoter region of the thyroid transcription factor-1 (TTF-1), which is essential for thyroid organogenesis, concurrently with increased dimethyl-H3-K9 and decreased acetyl-H3-K9, has been observed in a subset of thyroid carcinoma cells that had lost TTF-1 expression (Kondo et al., 2009); moreover, it has recently been demonstrated that the enhancer of zeste homolog 2 (EZH2), a histone lysine methyltransferase belonging to the polycomb group protein family, is specifically overexpressed in ATC, and it directly contributes to transcriptional silencing of *PAX8* gene and ATC differentiation (Borbone et al., 2011).

microRNA

Comparative analysis of miRs expression in normal thyroid tissue versus neoplastic tissue has shown that, in thyroid neoplasms, 32% of miRs were up-regulated and 38% were down-regulated; moreover, miRs expression profiles had substantial variability within specific tumor types (Nikiforova et al., 2008). Down-regulation of anti-invasive miR-200 and miR-30 families directs the EMT and invasive potential of ATCs (Braun et al., 2010). Recently it has been reported that two histone deacetylase inhibitors, trichostatin A and vorinostat, induced miR-129-5p overexpression, histone acetylation and cell death in papillary, and anaplastic cancer cell lines and in primary cultures of papillary thyroid cancer (Brest et al., 2011).

EPIGENETIC DRUGS

Epigenetic therapy was conceived to activate genes abnormally silenced in cancer by epigenetic mechanisms. Epigenetic drugs are expected to target the two main mechanisms of epigenetic alterations, DNA methylation, and acetylation, controlling through the differentiation and proliferation of transformed cells. To date, different epigenetic drugs are in clinical trials for the treatment of advanced thyroid cancer (Table 2).

The possibility of a microRNA-based therapy in cancer, using these small molecules as both targets and tools, suggests a new intriguing and promising therapy. Several preclinical studies underline this possibility; however, as to date no clinical trial is available, there will be no further discussion in the present review.

DEMETHYLATING AGENTS

During thyroid tumor progression, many specific thyroid genes (e.g., *NIS* and *TSH receptor* genes) are hypermethylated and, therefore silenced; demethylating agents might reverse the malignant

cell phenotype. Indeed, it was reported that demethylating agents like decitabine are able to restore NIS and TSH-R expression in human thyroid carcinoma cell lines (Venkataraman et al., 1999; Xing et al., 2003; Provenzano et al., 2007; Tuncel et al., 2007). Furthermore, decitabine treatment suppressed the growth of undifferentiated and dedifferentiated thyroid carcinoma cells (Miasaki et al., 2008; Vivaldi et al., 2009).

At present, the methyltransferase inhibitors azacitidine and decitabine have been licensed for clinical therapy only in myelodysplastic syndrome, but new hypomethylating agents (zebularine and isothiocyanates) are in various stages of development for cancer therapy (Kurkjian et al., 2008). In particular, a phase II clinical trial is ongoing for treatment with decitabine of patients with metastatic thyroid cancers unresponsive to radioiodine (see text footnote 2). The results of the trial, whose primary objective is to determine whether decitabine can restore iodine 131 uptake in patients with metastatic papillary thyroid or follicular thyroid cancer, are expected for June 2012.

HISTONE DEACETYLASE INHIBITORS

Histone acetylation and deacetylation are key events in controlling gene transcription; histone acetyltransferases (HATs) and histone deacetylases (HDACs; Kuo and Allis, 1998) catalyze these reactions and target also non-histone proteins, like transcription factors (Sterner and Berger, 2000).

HDAC inhibitors (DCI) are looked upon as very promising agents in tumor treatments since they, targeting multiple tumorigenic pathways, preferentially kill transformed cells, and are relatively non-toxic toward normal cells (Johnstone, 2002). Several structural classes of DCI have been identified, including short-chain fatty acids such as phenylbutyrate and valproic acid (VPA); cyclic tetrapeptides such as trapoxin A, cyclic peptides such as depsipeptide (FK228) and apicidin; benzamides such as MS27-275 and CI-994; hydroxamic acids such as suberoylanilide hydroxamic acid (SAHA), oxamflatin, trichostatin A, and the more recently developed pan-inhibitors LAQ824, PXD101, and LBH589 (Fuino et al., 2003; Plumb et al., 2003; Atadja, 2009).

In thyroid cancer, depsipeptide, SAHA, valproic acid, and panobinostat are the main DCI that are under investigation.

Depsipeptide

Depsipeptide (FK228 or FR901228) is a cyclic peptide that inhibits histone deacetylase activity at nanomolar concentrations (Johnstone, 2002). In SW-1736 cells it stimulates the expression of NIS, restores iodide uptake (Kitazono et al., 2001), and sensitizes cells to doxorubicin (Kitazono et al., 2002). The drug used alone inhibits the growth of a primary culture from a metastatic BRAF V600E papillary tumor; while in combination with paclitaxel, lovastatin, or gefitinib has synergic effects (Copland et al., 2006). p53 gene therapy associated with depsipeptide inhibits the growth of anaplastic FRO cell line (Imanishi et al., 2002).

There are currently two clinical studies at the National Cancer Institute (see text footnote 2). The first is a phase I trial examining the safety and tolerability of depsipeptide in patients with solid tumors, including thyroid cancer, which has recently been completed; the second is a phase II trial determining the anti-tumor activity of the drug in patients with progressive recurrent and/or

metastatic non-MTCs that are refractory to radioactive iodine. No conclusive data from these studies are available at the moment.

Vorinostat

Vorinostat (SAHA) is a hydroxamic acid that, binding directly to the HDAC catalytic site, inhibits DAC enzymatic activity (Finnin et al., 1999). SAHA induces growth arrest and caspase-mediated apoptosis in anaplastic thyroid cancer FRO cells (Mitsiades et al., 2005). Moreover it sensitizes cells to doxorubicin and inhibits the proliferation of FRO anaplastic tumors grown in nude mice (Luong et al., 2006).

In a phase I study on vorinostat in advanced cancers, six patients with thyroid cancer were recruited; one patient with advanced papillary tumor exhibited a partial response, while one papillary thyroid patient showed improvement in the RAI scan post-therapy (Kelly et al., 2005). Successively, a phase II trial was undertaken to determine whether SAHA was active in patients with metastatic thyroid cancers who did not respond to standard therapy, but unfortunately, it concluded that 200 mg of vorinostat twice a day was not a useful treatment for advanced thyroid cancer (Woyach et al., 2009).

Valproic acid

Valproic acid is a short-chain fatty acid which has been used for a long time in epilepsy and mood disorder treatment (Blaheta et al., 2005). More recently its activity as DCI (Göttlicher et al., 2001; Phiel et al., 2001) as well as its anti-proliferative and pro-differentiating properties in a variety of hematologic and solid tumors (Duenas-Gonzalez et al., 2008) were reported.

In the last decade, our laboratory was very interested in studying the anti-tumor effects of VPA on undifferentiated thyroid cancer cells. In 2004 we demonstrated that valproic acid induces NIS gene expression, NIS membrane localization, and iodide accumulation in poorly differentiated thyroid cancer cells (Fortunati et al., 2004). We also showed (Catalano et al., 2005) that the drug is highly effective in suppressing the growth of poorly differentiated thyroid cancer cell lines, inducing apoptosis and cell cycle. In agreement with our results, Shen et al. (2005) demonstrated that, also in metastatic follicular cell lines, VPA causes significant growth inhibition. Unfortunately, in ATC cells, the induction of NIS mRNA is not followed by a change in iodide uptake (Fortunati et al., 2004) and VPA does not induce apoptosis (Catalano et al., 2006). Taken together, these data indicate that VPA alone is not effective in anaplastic thyroid cancer; however, we demonstrated that it enhances the cytotoxicity of doxorubicin in ATC cells and that the sensitizing effect increased apoptosis and doxorubicin-induced G₂ cell cycle arrest (Catalano et al., 2006). Afterward, it has been reported that some cell lines employed in those *in vitro* studies are of non-thyroid origin (Schweppe et al., 2008); thus, results obtained using these cell lines need to be regarded with caution. However in the same studies, cells of assessed thyroid origin showed a similar behavior.

In line with these findings, it has been reported that VPA, in combination with the highest concentration of doxorubicin that does not induce KAT-18 cell death, efficiently induced apoptosis in KAT-18 cells (Kim et al., 2009). Since some DCI that target HDAC6 (Zhang et al., 2003), like VPA, can also have α -tubulin as a substrate

and tubulin acetylation is linked to microtubule dynamics and stability (Matsuyama et al., 2002), the effect of VPA used together with paclitaxel, a microtubule-targeting drug, were evaluated. The addition of VPA to paclitaxel significantly enhanced the cytostatic and cytotoxic effects of paclitaxel in ATC cells (Catalano et al., 2007). Beside the *in vitro* studies, one case of successful treatment of ATC with a combination of oral VPA, cisplatin and doxorubicin, plus external and intra-operative radiation, and surgery has been reported the patient being alive and disease-free 2 years after diagnosis (Noguchi et al., 2009). A phase II trial of VPA in patients with advanced thyroid cancers of follicular origin is active and currently recruiting patients at the NCI (see text footnote 2). Moreover, an Italian multicenter phase II/III clinical trial in patients with undifferentiated thyroid cancer treated with a combination of VPA and paclitaxel is ongoing³.

Panobinostat

Panobinostat (LBH589) is a hydroxamic acid with potent inhibitory activity at low nanomolar concentrations against all classes of HDAC enzymes (pan-DAC inhibitor; Atadja, 2009). *In vitro* treatment with LBH589 of three ATC cell lines (BHT-101, CAL-62, and 8305C) resulted in impairment of cell viability, inhibition of colony formation, cell cycle arrest, and apoptosis induction. LBH589 markedly determined microtubule stabilization as evidenced by tubulin acetylation and increased

³<https://oss-sper-clin.agenziafarmaco.it>

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Role of the Wnt pathway in thyroid cancer

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Aberrant activation of Wnt signaling is involved in the development of several epithelial tumors. Wnt signaling includes two major types of pathways: (i) the canonical or Wnt/β-catenin pathway; and (ii) the non-canonical pathways, which do not involve β-catenin stabilization. Among these pathways, the Wnt/β-catenin pathway has received most attention during the past years for its critical role in cancer. A number of publications emphasize the role of the Wnt/β-catenin pathway in thyroid cancer. This pathway plays a crucial role in development and epithelial renewal, and components such as β-catenin and Axin are often mutated in thyroid cancer. Although it is accepted that altered Wnt signaling is a late event in thyroid cell transformation that affects anaplastic thyroid tumors, recent data suggest that it is also altered in papillary thyroid carcinoma (PTC) with RET/PTC mutations. Therefore, the purpose of this review is to summarize the main relevant data of Wnt signaling in thyroid cancer, with special emphasis on the Wnt/β-catenin pathway.

Keywords: Wnt pathway, signaling network, thyroid cancer, proliferation, differentiation

INTRODUCTION

Follicular epithelial thyroid carcinoma is the most common endocrine malignancy, and its incidence is rapidly rising in the world. Although thyroid cancer has a very good outcome, there are still several challenges that classical clinicopathological studies have not been able to resolve (Riesco-Eizaguirre and Santisteban, 2007). The most recent advances in thyroid cancer research derive from an increased understanding of the mechanisms that regulate thyroid cell differentiation and proliferation and the signal transduction pathways involved.

Differentiated thyroid cells form follicular structures surrounding a lumen and express a series of thyroid-specific transcription factors as well as other specific proteins (reviewed in De Felice and Di Lauro, 2004; Santisteban and Bernal, 2005). Thyroid proliferation and differentiation are finely regulated by Thyrotropin (thyroid stimulating hormone, TSH) that, after binding to its receptor (TSHR, a seven-transmembrane receptor coupling to G proteins), activates the cAMP/PKA cascade (Vassart and Dumont, 1992). Recently our group has shown that TSH can also act via PI3K/Akt (Zaballos et al., 2008). In addition, increasing evidence supports a role for other kinases, such as MAP kinase (MAPK), which are activated in response to TSH and lie downstream of cAMP (Dumaz and Marais, 2005).

The differentiated thyroid epithelial phenotype is characterized by structural and functional polarization of the cell surface into apical and basolateral domains, and the formation of junction complexes that mediate strong and tight intercellular adhesion. Cell polarization plays a central role in maintaining follicular structures and its alteration occurs in parallel with cell transformation. The presence of tight junctions in thyroid epithelial cells

is thought to be essential for the integrity and maintenance of the follicular structure. Several molecules are involved in this process; of these, the occludins, claudins, and zonula occludens (ZO) proteins have been most intensively studied, as they play an important role in the maintenance of the epithelial cell phenotype. As occurs with other epithelial markers, their expression is reduced in thyroid cancer progression (Tzelepi et al., 2008). On the other hand, adherens junctions are mainly composed of cadherins. Among them, E-cadherin and Cadherin 16 are highly expressed in the thyroid (Cali et al., 2012). Both are members of the large superfamily of adhesion molecules and play a critical role in the establishment of cell polarity and firm contacts.

Adherens junctions have a similar structural organization as tight junctions: E-cadherins and Cadherin 16 form contacts with the catenins (α, β, and γ) and αB-crystallin respectively, and these latter proteins connect with the cytoskeleton (Cali et al., 2012). The follicular structure is maintained by a series of proteins through which TSH and intercellular contact regulate adhesion of follicular cells to each other and to the extracellular matrix, and influence thyroid cell behavior. The extracellular matrix plays a role in the adhesion, proliferation, differentiation, and migration of thyroid follicular cells.

It has been reported that downregulation of cadherins takes place in carcinogenesis and is associated with tumor progression in a variety of human carcinomas (Strumane et al., 2004; Berx and van Roy, 2009). Therefore, decreased expression or loss of cadherins may lead to the development of epithelial tumors, invasion, and metastasis. In accordance with this, the loss of E-cadherin and Cadherin 16 expression in thyroid carcinoma has been correlated with a loss of differentiation and a poor prognosis (Brabant et al., 1993; Cali et al., 2012; de Cristofaro et al., 2012).

THYROID CANCER INITIATION AND PROGRESSION

Thyroid cell proliferation requires the combined effects of TSH, acting via cAMP, and growth factors such IGF1, signaling primarily through MAPK and phosphatidylinositol-3-kinase (PI3K; Medina and Santisteban, 2000; Kimura et al., 2001). It is therefore well accepted that mutations in genes involved in these signaling pathways play prominent roles in the pathogenesis of thyroid neoplasia.

Currently there are two hypotheses to explain the onset of thyroid cancer, the classical multistep model and a theory based on cancer stem cells (CSCs).

The classical view considers thyroid carcinoma as a complication of a pre-existing follicular adenoma (FA) accumulating mutations that drive progression through a dedifferentiation process. Accordingly, a step model of thyroid carcinogenesis involving different defined types of thyroid tumor is well accepted (Figure 1). Thus, autonomously hyperfunctioning thyroid adenomas and FAs are benign tumors that rarely progress to malignancy. Papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC) show follicular cell differentiation, and poorly differentiated carcinomas (PDC) have morphological and biological characteristics intermediate between well-differentiated and undifferentiated (anaplastic) thyroid carcinomas (ATC; reviewed in Riesco-Eizaguirre and Santisteban, 2007, and in Nikiforov and Nikiforova, 2011).

The genetic events involved in tumor initiation have been identified in well-differentiated thyroid cancer. In PTC there are many data supporting a central role of mutations leading to constitutive activation of the MAPK pathway. Thus, the V600E mutation of BRAF, RET/PTC rearrangements, and RAS mutations have been clearly implicated in the pathogenesis of this disease. These three pathways are mutually exclusive and it is accepted that they are the cause of a significant proportion of PTCs. Similarly, there is increasing evidence that shows that cancer genes acting through

the PI3K pathway (PI3KCA and PTEN) are involved in the pathogenesis of FTC. However, in this case more studies are needed to elucidate a possible causal effect of these genetic events. Furthermore, a PAX8/PPAR γ rearrangement (Kroll et al., 2000) has been identified in a significant proportion of these tumors, although the mechanism of transformation induced by PAX8/PPAR γ is still unclear.

Deregulation of the p53 pathway seems to be an important second step leading to the progression of PDC/ATC (Fagin et al., 1993; Figures 1 and 2). In addition, activating mutations in the β -catenin gene (CTNNB1) have been described in ATC that occur late in thyroid tumor progression. Therefore, until the recent demonstration that RET/PTC stimulates the β -catenin pathway in the thyroid (Cassinelli et al., 2009; Castellone et al., 2009; Tartari et al., 2011), it was believed that β -catenin was not involved in thyroid tumor initiation. Compared with the well-documented RAS/RAF/MAPK pathway, the β -catenin signal in thyroid cancer is less well understood, and therefore in this review we will focus on updating the existing data and comment on future directions of research.

Recently, a new theory on the cancer initiation process was formulated, based on the existence of CSCs (Thomas et al., 2008; Lin, 2011). These CSCs are a small subpopulation (between 1 and 3.5%) of the cells with stem cell-like properties such as colony formation, self-renewal, and *in vitro* resistance to chemotherapy-induced apoptosis (Dick, 2008, 2009). In the case of the thyroid gland, the CSCs would derive from thyroid embryonic stem cells, intermediates in the differentiation pathway of thyroblasts or prothyrocytes, and accumulate mutations that lead to carcinogenesis. The CSC hypothesis assumes the presence of a hierarchy of embryonic cells in the thyroid gland that can give rise to different forms of thyroid cancer (reviewed in Lu et al., 2011).

The existence of CSCs in the thyroid is still a matter of controversy, but several lines of evidence support the model. Among them is the observation that it is uncommon that a benign adenoma

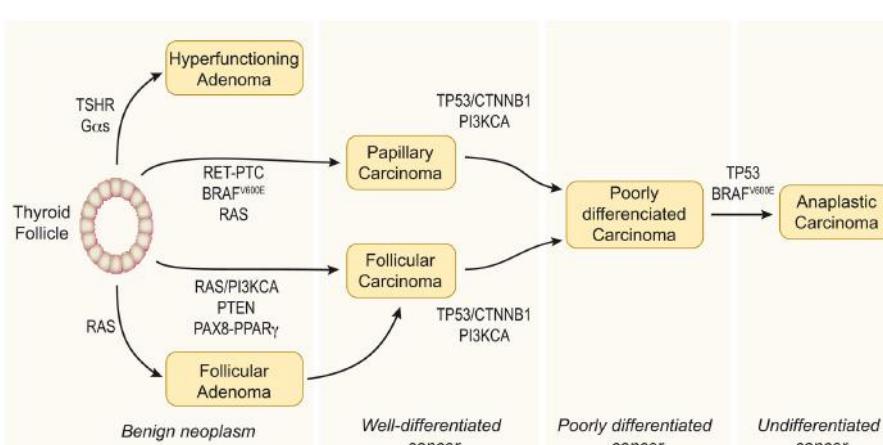


FIGURE 1 | Step model of thyroid carcinogenesis. This model is based on histological and clinical features as well as on the degree of tumor differentiation. The well-differentiated thyroid follicular cell may give rise to both benign and malignant tumors. Autonomously hyperfunctioning thyroid adenomas are associated with activating mutations in the *TSHR* or *Gαs* genes (O'Sullivan et al., 1991; Parma

et al., 1993). After gaining mutations in different oncogenes and tumor suppressor genes, the differentiated thyroid follicle can also give rise to well-differentiated papillary or follicular carcinomas, poorly differentiated carcinoma, and anaplastic carcinoma. The figure represents a schematic model showing the molecular events involved. Modified from Nikiforov and Nikiforova (2011).

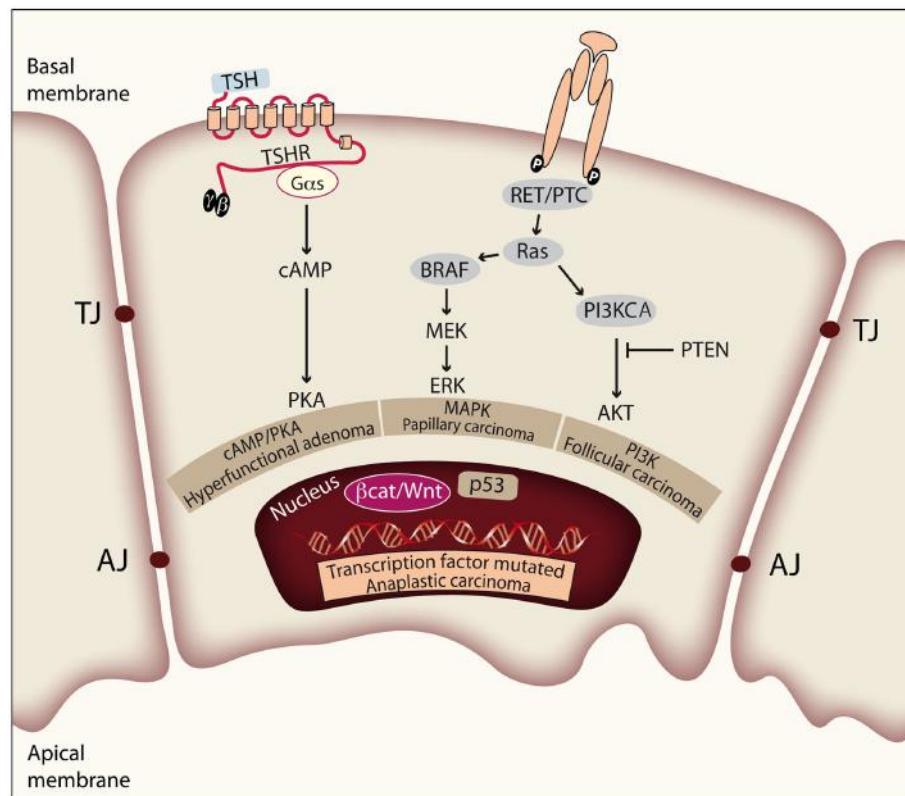


FIGURE 2 | Genetic events involved in thyroid tumor initiation and progression. Thyroid cell proliferation depends on the combined activation of cAMP/PKA, MAPK, and PI3K pathways, induced by TSH and other growth factors. Mutations of effectors along these signaling pathways play prominent roles in the pathogenesis of thyroid neoplasia. The figure represents a thyroid epithelial cell with the basal and apical membrane, and shows the tight and adherens junctions (TJ and AJ) between cells in order to

form follicular structures. Activating mutations in *TSHR* or *Gαs* lead to constitutive activation of the cAMP/PKA pathway and give rise to hyperfunctional adenomas. There is evidence that constitutive activation of the MAPK pathway is required for PTC initiation. Activation of the PI3K/Akt pathway is required for FTC initiation. Finally, deregulation of the p53 pathway and the Wnt/β-catenin pathway is observed in ATC and has more to do with tumor progression.

evolves toward carcinoma; at present, it seems that most thyroid carcinomas are malignant from the onset; the original *RET/PTC* mutations seen in FTC and PTC are hardly seen in ATC. In addition, data from Chernobyl irradiation studies, in which the highest incidence of papillary thyroid cancer was among children rather than adults, strongly support the CSC theory. The establishment of ES cell cultures able to differentiate into thyrocytes may help clarifying these views (Arufe et al., 2006).

Wnt SIGNALING

Wnt proteins are a family of highly conserved secreted cysteine-rich glycoproteins, encoded by 19 genes in humans and 18 genes in mice. These proteins play important roles in embryonic development by controlling cell proliferation, cell fate specification, tissue patterning, and cell polarity. In adults, they contribute to tissue homeostasis by controlling proliferation, stem cell activation, and self-renewal. Signaling by Wnt proteins activates three different pathways: one canonical or β-catenin-dependent pathway and two non-canonical or β-catenin-independent pathways: Wnt/Ca²⁺ and planar cell polarity (PCP; Figure 3). The relevance of these pathways is also reflected by the fact that mutations in some elements of the Wnt pathways are often linked to human

diseases like leukemia and other cancers, and type-II diabetes (Kikuchi and Yamamoto, 2008; MacDonald et al., 2009).

CANONICAL Wnt/β-CATENIN PATHWAY

The most extensively studied Wnt pathway is the Wnt/β-catenin pathway, due to its important role in cancer initiation and progression (Reya and Clevers, 2005; see Figure 3, left). In the absence of Wnt, β-catenin, the central component of the pathway, is localized in adherens junctions bound to E-cadherin. The unbound cytoplasmic protein is constantly degraded by a protein complex called destruction complex, which is composed of the scaffold proteins Axin and adenomatous polyposis coli (APC), and the kinases casein kinase 1 (CK1) and glycogen synthase kinase 3 beta (GSK3β; Rubinfeld et al., 1996). CK1 and GSK3β sequentially phosphorylate β-catenin, resulting in β-catenin being recognized and ubiquitinated by the β-Trcp ubiquitin ligase, followed by proteasomal degradation. With low levels of free cytoplasmic β-catenin, the transcription factors T-cell factor/lymphoid enhancer factors (TCF/LEF) function as transcriptional repressors by recruiting corepressors of the TLE/Groucho family.

The Wnt pathway is activated when a protein of the family, such as Wnt1, Wnt3, Wnt3a, Wnt7A, or Wnt10B, binds

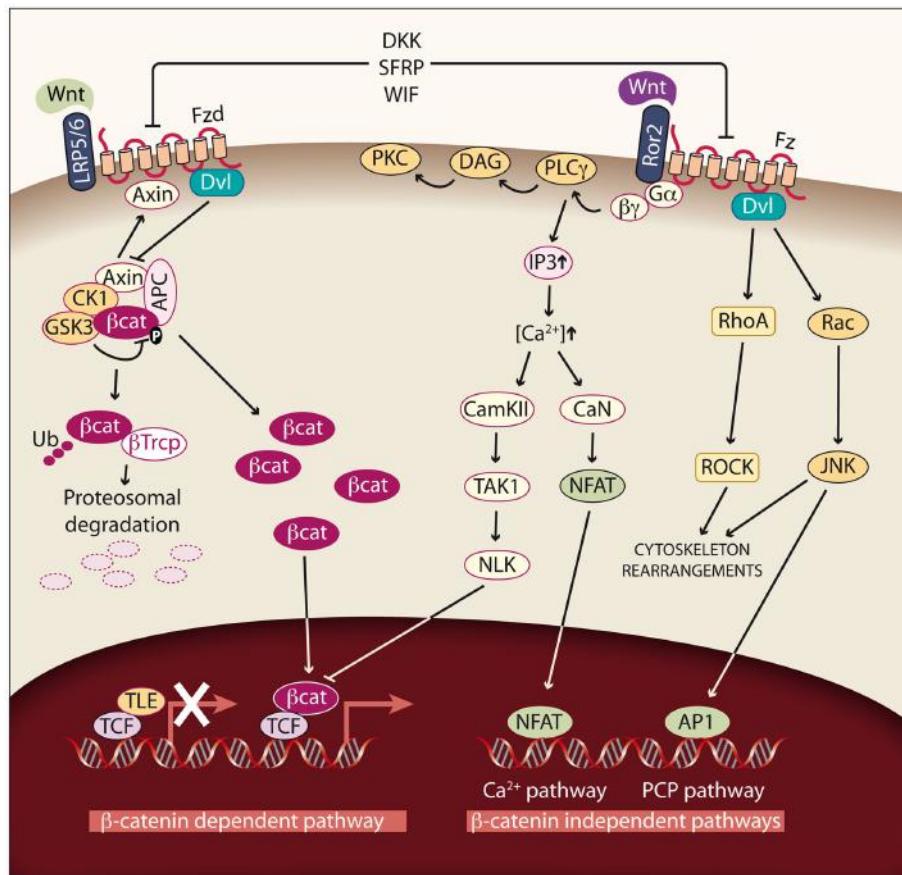


FIGURE 3 | Wnt signaling pathways. *Left:* Canonical or Wnt/β-catenin dependent pathway. In cells not exposed to Wnt factors, cytoplasmic β-catenin is degraded, and TLE/Groucho proteins repress its target genes. If Wnt signaling is activated by binding of Wnt factors such as Wnt1, Wnt3, Wnt3A, Wnt7A, or Wnt10B to the Fzd receptor and LRP5/6 coreceptors, β-catenin degradation is reduced and the protein accumulates in the cytoplasm. As a consequence, β-catenin enters the nucleus, binds to TCF/LEF transcription factors and activates transcription. In this way, different processes such as proliferation are modulated. *Right:*

Non-canonical or β-catenin-independent pathways. Binding of the non-canonical Wnt factors Wnt4, Wnt11, or Wnt5A to different Fzd receptors and the Ror2 coreceptor transduce signaling by two different pathways. The Ca^{2+} pathway promotes the activation of protein kinase C (PKC) via G-PLC γ and modulates cell adhesion and motility by activating calcium-calmodulin kinase (CamKII), and the phosphatase calcineurin (CaN). The planar cell polarity (PCP) pathway modulates cytoskeleton rearrangements through the activation of the small GTPases RhoA and Rac and their downstream effectors Rock and JNK.

to a frizzled receptor (Fzd) and the LDL-Receptor-related protein coreceptor (LRP5/6). Formation of a Wnt–Fzd–LRP complex induces the binding of the cytoplasmic protein Disheveled (Dvl) to Fzd and the LRP phosphorylation-dependent recruitment of Axin to the membrane. Recruitment of Axin, which is the limiting component of the destruction complex, promotes the release of β-catenin, and its accumulation in cytoplasm and nuclei. In the nuclei, β-catenin displaces TLE/Groucho corepressors and recruits coactivators, activating expression of Wnt target genes. The meanings of the acronyms used for the different members of the Wnt pathway are given in Table 1; see <http://www.stanford.edu/group/nusselab/cgi-bin/wnt/> for more details.

The most important genes regulated are those related to proliferation, such as *Cyclin D1* and *c-Myc* (He et al., 1998; Tetsu and McCormick, 1999), which are over-expressed in most β-catenin-dependent tumors.

NON-CANONICAL Wnt PATHWAYS

There are two main β-catenin-independent pathways (see Figure 3, right). The activation of these pathways is mediated by Wnt factors such Wnt4, Wnt11, and Wnt5A in a tissue and context specific way. The first pathway is the PCP pathway. This pathway can be triggered through several of the Fzd receptors and it activates the small GTP-binding proteins RhoA and Rac and their downstream effectors Rho-kinase and JNK. This non-canonical pathway is involved in tissue polarity, cell migration, and cytoskeleton organization. It has been described in *Drosophila*, and although its relevance in mammals has not been fully demonstrated, there is important evidence pointing to its existence in these vertebrates (Wada and Okamoto, 2009). The second pathway is the Ca^{2+} pathway, which can be activated by the interaction with two Fzd receptors (Fzd 2 and 7) or with Ror2 (receptor tyrosine kinase-like orphan receptor 2). The activation of this pathway leads to an activation of PKC and to increased intracellular Ca^{2+} levels,

Table 1 | Members of the Wnt pathway grouped according to function.

β-Catenin destruction complex	APC1/2 (adenomatous polyposis coli) Axin 1/2 GSK3β (glycogen synthase kinase 3 beta) CK1 (casein kinase 1)	
Receptor/co-receptors	Fzd 1–10 (frizzled) LRP5/6 (LDL receptor protein) co-receptor Ror2 (receptor tyrosine kinase-like orphan receptor 2) Dvl 1–3 (dishevelled: cytoplasmic protein downstream of Fzd receptors)	
Wnt ligands	β-catenin-dependent pathway activators β-catenin-independent pathways activators	Wnt1, 2, 2B, 3, 3A, 6, 5B, 7A, 7B, 8A, 8B, 9A, 9B, 10A, 10B, 16 Wnt4, 5A, and 11
Transcription factors	TCF1, 3, and 4; Lef1 (T-cell factors; lymphoid enhancer factor)	
Transcriptional co-activators	β-catenin	
Transcriptional co-repressors	TLE (transducin-like enhancer of split)/Groucho 1–4	
Wnt inhibitors	DKK 1–4 (dickkopf) SFRP 1–5 (secreted frizzled-related proteins) Soggy and WIF	

which activates CaMKII and calcineurin (CaN) and regulates cell migration and proliferation (Nishita et al., 2010).

Wnt INHIBITORS

Activation of the Wnt pathway is highly regulated, and there are two families of antagonists: DKK and secreted frizzled-related proteins (SFRP). The Dickkopf (DKK) family is a group of four soluble proteins that bind to LRP and thus prevent the formation of the Wnt–Fzd complex. The SFRP are soluble glycoproteins that inhibit the Wnt pathway by sequestering Wnt proteins, thus preventing their interaction with Fzd receptors. There are other Wnt inhibitors like WIF or Soggy1 (Filipovich et al., 2011; **Figure 3**). The physiological role of these proteins is to inhibit the Wnt pathway in a time and tissue-specific manner both during development and in adult tissues. The silencing of DKK and SFRP genes has been described in several tumor types, contributing to the activation of the canonical pathway (Aguilera et al., 2006).

Wnt AND THYROID CANCER

Normal thyroid cells express several Fzd, Dvl, and Wnt proteins and have a functional destruction complex (Helmbrecht et al., 2001). In addition, TSH-dependent over-expression of Wnt1 (Kim et al., 2007) and inhibition of GSK3β by adenoviral-interference (Chen et al., 2010) lead to an increase of rat and human thyroid cell proliferation. These data suggest the existence of a functional Wnt pathway, relevant for the proliferation of thyroid cells.

Given the hypothetical role of this pathway in proliferation, it is not surprising that over-activation of the Wnt/β-catenin pathway promotes tumor growth in the thyroid, as in other Wnt-dependent tissues like skin or colon.

Classically, activation of the Wnt pathway in thyroid cancer has been related with ATCs, as a second mutational event involved in the progression from a well-differentiated to a poorly or undifferentiated (anaplastic) and more aggressive thyroid carcinoma. However, as commented above, recent data correlate this pathway also with early stages of thyroid carcinogenesis.

Wnt IN POORLY DIFFERENTIATED AND ANAPLASTIC THYROID CARCINOMA

Mutations of several components of the Wnt pathway have been described in ATC (**Table 2**). The most frequent mutations are those in the scaffold proteins APC and Axin, as well as in β-catenin (Polakis, 2007). In tumors such as colon tumors mutations most frequently affect the APC gene, but in the thyroid the mutations appear mainly in β-catenin and Axin.

In ATC, three different studies found nuclear localization of β-catenin in 40–60% of the samples analyzed, highlighting the role of the Wnt pathway in this type of tumor (Garcia-Rostan et al., 1999, 2001; Kurihara et al., 2004). In these studies, performed in three different human populations, mutations in the β-catenin and Axin1 genes were described. In two of them, all β-catenin mutations found were in the conserved Ser and Thr residues phosphorylated by CK1 and GSK3β, which results in a constitutive stabilization of the protein and successive accumulation in both cytoplasm and nucleus. The presence of nuclear β-catenin correlated with a higher proliferation and a loss of tumor differentiation, and therefore with a poor prognosis (Garcia-Rostan et al., 1999, 2001). Despite the role of β-catenin in proliferation, these latter two studies together with another one (Cerrato et al., 1998) also described a percentage of anaplastic tumors in which the β-catenin expression is absent.

These data can be interpreted to mean that there are distinct subtypes of tumors in ATC with different, mutually exclusive mutations. Thus, one subtype would carry the Wnt/β-catenin pathway mutation, while the other would carry the p53 mutation (Fagin et al., 1993) or an activated PI3K/Akt pathway, e.g., through a mutation in PI3KCA (Garcia-Rostan et al., 2005); these three are the most frequent mutations found in ATC. It would be of great interest to establish whether mutations in oncogenes or tumor suppressor genes are mutually exclusive in order to better classify anaplastic thyroid tumors.

By contrast, the study by Kurihara et al. (2004) described a low percentage of β -catenin mutations, while more than 50% of the tumors carried mutations in the functional domain of Axin1. The different mutations found in the *Axin1* gene affect the domains for interaction with APC, β -catenin and Dvl, and the G-protein regulatory domain, and thus affect the role of Axin as a negative regulator of β -catenin. The difference in data regarding β -catenin and Axin mutations may be due to the different genetic backgrounds of the populations analyzed.

Wnt IN WELL-DIFFERENTIATED THYROID CARCINOMA

It is well accepted that there is a correlation between the subcellular localization of β -catenin and cancer progression (Garcia-Rostan et al., 2001). In hyperfunctioning adenomas and FTC, β -catenin is localized in the plasma membrane, as it is in the normal thyroid gland. In PTC, there is an accumulation of β -catenin in the cytoplasm, and in poorly and undifferentiated carcinoma β -catenin is translocated to the nuclei due to mutations in the β -catenin

gene as well as in other genes of the Wnt pathway (Table 3). We already discussed the role of β -catenin in ATC, but the role and the molecular mechanisms that lead to the cytoplasmic stabilization of β -catenin have not been established. Some groups have correlated cytoplasmic β -catenin in PTC with higher levels of cyclin D1 (Ishigaki et al., 2002; Meirmanov et al., 2003; Rezk et al., 2004; Zhang et al., 2011) and increased proliferation. Nevertheless, at present there are no *in vitro* results that corroborate this hypothesis and no relationship between cytoplasmic β -catenin and transcriptional activity has been found. More studies are needed to demonstrate a direct link between cytoplasmic β -catenin and cyclin D1 expression in order to correlate both events with proliferation.

E-cadherin is a transmembrane protein that mediates cell–cell adhesion in a Ca^{2+} -dependent manner. It interacts through its cytoplasmic domain with β -catenin and the actin cytoskeleton, controlling cell migration and cell polarity (Hulsken et al., 1994). In normal thyroid cells, E-cadherin is expressed in the basolateral

Table 2 | Events linked with aberrant activation of Wnt signaling in thyroid cancer.

Gene	Mutation or activity/expression	Tumor	No. of cases	Reference
CTNNB1*	GOF	ATC	19/31	Garcia-Rostan et al. (1999)
		ATC (PD)	7/28	Garcia-Rostan et al. (2001)
		ATC (UD)	19/29	Garcia-Rostan et al. (2001)
Axin 1	LOF	ATC	18/22	Kurihara et al. (2004)
APC	LOF	CMV-PTC	4/4	Cetta et al. (1998)
			15/15	Cetta et al. (2000)
Wnt5A	Elevated	FTC/PTC	8/8–10/11	Kremenevskaja et al. (2005)
Wnt5A	Reduced	ATC	5/5	Kremenevskaja et al. (2005)

* β -catenin gene; GOF, gain of function; LOF, loss of function; FTC, follicular thyroid carcinoma; PTC, papillary thyroid carcinoma; ATC, anaplastic thyroid carcinoma; PD, poorly differentiated; UD, un-differentiated; CMV-PTC, cribriform–morular variant of PTC.

Table 3 | Localization of β -catenin in thyroid carcinoma.

Tumor type	Cytoplasmic expression	Nuclear expression	No. of cases	Reference
FA	3 (9%)		34	Ishigaki et al. (2002)
	3 (37.5%)		8	Meirmanov et al. (2003)
FTC	5 (25%)		20	Ishigaki et al. (2002)
	8 (80%)		10	Rezk et al. (2004)
	8 (60%)		12	Garcia-Rostan et al. (2001)
PTC	46 (100%)		46	Garcia-Rostan et al. (2001)
	46 (87%)		53	Rezk et al. (2004)
	52 (67%)		78	Ishigaki et al. (2002)
	23 (100%)		23	Meirmanov et al. (2003)
FVPTC	33 (71%)		46	Garcia-Rostan et al. (2001)
ATC (PD)			28	Garcia-Rostan et al. (2001)
ATC (UD)			29	Garcia-Rostan et al. (2001)
ATC		15 (41%)	36	Garcia-Rostan et al. (1999)
	10 (49%)	14 (63.6%)	22	Kurihara et al. (2004)

FA, follicular adenoma; FVPTC, follicular variant of PTC; the other acronyms of tumor type as defined in Table 2.

membrane and its downregulation, by promoter methylation or by activation of oncogenes such as *BRAF*, has been implicated in the induction of the epithelial mesenchymal transition (EMT) in follicular, papillary, and anaplastic thyroid tumor cells (Brabant et al., 1993; Graff et al., 1998; Riesco-Eizaguirre et al., 2009). As E-cadherin keeps β -catenin bound to the cell membrane, the presence of cytoplasmic β -catenin could be merely a consequence of the loss of E-cadherin expression, although this correlation has not yet been demonstrated.

Supporting the role of β -catenin in earlier stages of tumor progression, recent publications describe the involvement of β -catenin in RET/PTC-induced proliferation (Cassinelli et al., 2009; Castellone et al., 2009; Tartari et al., 2011). These studies show a RET/PTC-dependent stabilization of β -catenin by phosphorylation of a residue outside the GSK3 β Ser/Thr domain. This stabilization, together with an Akt–MAPK-dependent inhibition of GSK3 β , leads to an increase of β -catenin in the nuclei where it is able to interact with the transcription factors TCF/LEF and CREB binding to the *cyclin D1* promoter. In consequence, DNA synthesis and cell proliferation are induced. This is another way of Wnt/ β -catenin pathway regulation, by post-translational modifications, that points to a participation of this pathway in the first steps of thyroid cell transformation. Unfortunately, the authors did not correlate their results with the localization of β -catenin in RET/PTC-carrying thyroid tumors.

Data obtained from the TR β ^{PV/PV} mouse model of FTC that harbors a dominant negative mutation (PV) of the thyroid hormone-beta receptor (TR β), also support the notion that β -catenin could contribute to thyroid carcinogenesis (Guigon et al., 2008; Lu et al., 2011). Thus, in the FTC tumors of these mice thyroid hormone and its receptors seem to modulate the Wnt/ β -catenin pathways in two ways. First, the TR β acts as negative regulator of β -catenin in a T3-dependent manner (Guigon et al., 2008). In the absence of ligand, TR β binds to β -catenin and stabilizes it in the cytoplasm and the nuclei allowing β -catenin to operate as a transcriptional activator promoting cell proliferation. Binding of T3 to TR β weakens the physical interaction between β -catenin and TR β and allows the uncomplexed β -catenin to be targeted for proteasomal degradation. In the thyroid tumors developed in the transgenic TR β ^{PV/PV} mice, β -catenin is constitutively stabilized through its binding to the mutant TR β , because the mutated TR β cannot bind T3. In this way, β -catenin activates the expression of its target genes *cyclin D1*, *myc*, and *MT1-MMP* (matrix metalloproteinase), and promotes tumor growth and progression. The second mechanism involves a non-genomic action: T3 (elevated in the TR β ^{PV/PV} mice) is able to increase the PTEN/PI3K/Akt pathway through the $\alpha v \beta 3$ receptor, leading to the phosphorylation of β -catenin, which increases its stability and transcriptional activity and promotes cell proliferation (Lu et al., 2011). Overall, these results show a new mechanism of activation of the Wnt/ β -catenin pathway, which through the stabilization of β -catenin could be promoting cancer progression in the thyroid gland.

All the above evidence points to a role of β -catenin in well-differentiated thyroid carcinomas, but more *in vitro* and *in vivo* evidence is needed and the molecular mechanism remains to be resolved.

NON-CANONICAL Wnt PATHWAYS IN THYROID CARCINOMAS

Wnt5A is an activator of the non-canonical Wnt pathways. Due to its roles in planar polarity and cell migration and invasiveness, Wnt5A has been implicated in several human cancers, but, as occurs in normal tissues, the roles of Wnt5A in cancer are tissue and receptor specific. In osteosarcoma as well as in prostate and renal cell carcinomas, Wnt5A acting through the Ror2 receptor is involved in matrix metalloprotease expression, enhancing cell migration and invasiveness of these cells, and consequently it is a poor prognosis factor for these types of tumors. By contrast, in colon and thyroid tumors Wnt5A acts as a tumor suppressor, highlighting the variety of roles of this Wnt member (McDonald and Silver, 2009).

While Wnt5A is expressed in FA, PTC, and FTC, no expression has been detected in ATC or in the normal thyroid (Kremenevskaja et al., 2005). In well-differentiated cancer cells, Wnt5A acts as a tumor suppressor by inhibiting both Wnt/ β -catenin-dependent proliferation in a Ca²⁺/CaMKII dependent manner, and migration and invasiveness. In this way, it promotes a mesenchymal epithelial transition (MET) by the induction of cadherin expression and the re-localization of β -catenin from the nuclei to the membrane. Wnt5A expression is lost in anaplastic carcinoma, leading to a more aggressive tumor in which proliferation, migration, and invasiveness are enhanced.

Wnt IN FAMILIAL SYNDROMES

Sporadic mutation of the *APC* gene is less frequent in thyroid tumors, but there is a high frequency of PTC in several syndromes carrying *APC* mutations, such as familial adenomatous polyposis (FAP), Gardner's syndrome, and Turcot's syndrome (Soravia et al., 1999). FAP arises as a consequence of germinal mutations in one allele of the *APC* gene. Patients develop mainly colonic polyps and non-colonic malignancies, the most frequent being PTC. These PTCs have characteristic histological structures and are called the Cribriform–Morular Variant of PTC (CMV of PTC), because of their morula-like structure. Such structures are frequent in other tumors and correlate with nuclear β -catenin and activation of the Wnt/ β -catenin pathway. Contrary to colorectal neoplasms that exhibit a loss of heterozygosity in the *APC* gene, the CMV of PTC maintains heterozygosity and seems to need other gene alterations to originate a tumor. RET–PTC rearrangements have been described as a frequent event in CMV PTC-FAP tumors, further suggesting that *APC* mutation alone is not sufficient as a tumor initiator (Cetta et al., 1998, 2000).

Mutations in the phosphorylation sites of β -catenin are also found in the CMV of PTC, where nuclear localization of β -catenin correlates with poorly or undifferentiated tumors. In one study it was suggested that a mutation in exon 3 of the β -catenin gene, *CTNNB1*, could be an early molecular event in the CMV of PTC, although mutations in other genes frequently altered in PTC, such as *RET*, *RAS*, or *BRAF* were not studied (Xu et al., 2003).

CANCER STEM CELLS AND THE Wnt PATHWAY

Wnt proteins contribute to the homeostasis of several tissues of epithelial origin, like intestine and skin. This is because activation of the Wnt pathway is absolutely required for driving the stem cell/progenitor compartment. This pathway is altered in thyroid

malignancies, together with other stem cell-regulating pathways such as Hedgehog and Notch signaling, which supports the CSC model (reviewed in Derwahl, 2011).

In fact, it is well accepted that the Wnt/β-catenin pathway is also necessary for the maintenance of CSCs. Thus, in colon the inappropriate activation of this pathway by *APC* or *β-catenin* gene mutations promotes the growth of tumor cells exhibiting a stem cell-like expression profile (Vermeulen et al., 2010). In breast cancer, the Wnt pathway is upregulated in CSCs by Wnt ligands secreted by the tumor microenvironment (Malanchi et al., 2011). Concerning thyroid CSCs there are still few data, but increasing evidence supports the existence of CSCs and the role of the Wnt pathway in this gland. Recent data demonstrate that CSCs from different types of thyroid carcinomas (PTC, FTC, and ATC), have different properties: CSCs isolated from ATC are the most aggressive and tumorigenic, followed by CSCs from PTC and FTC (Todaro et al., 2010). These data demonstrate that the malignancy of the CSCs correlates with the characteristics of the tumor and could reconcile the multistep process of thyroid carcinogenesis with the CSC hypothesis (Derwahl, 2011). Interestingly, the data reported by Todaro et al. showed constitutive activation of cMet, Akt, and β-catenin, together with downregulation of E-cadherin, in CSCs derived from the most undifferentiated thyroid tumors. This correlated with a higher migration capacity and metastatic rate. Although the above results are very promising, further studies are needed to evaluate the activation of the Wnt pathway and its role in CSC stemness or maintenance.

THE Wnt PATHWAY AS A TARGET FOR THYROID CANCER TREATMENT

Thyroid cancer has, in general terms, a very good outcome as radioiodine treatment is a very effective therapy. However, there are still some critical challenges that the classical clinicopathological approach has not been able to solve, and in some poorly differentiated radioiodine-resistant tumors and in ATC treatment options are limited.

In the past years, new drugs such as tyrosine kinase inhibitors or MAPK inhibitors proved to be quite efficient. The findings regarding the involvement of the Wnt pathway in thyroid cancer, its crosstalk with tyrosine kinase receptors such as RET and the involvement of β-catenin and Axin in ATC suggest that this pathway may be a potential therapeutic target. Current therapies with tyrosine kinase inhibitors such as Imatinib or Vandetanib seem to work in part by inhibiting the Wnt/β-catenin pathway. Treatment with Imatinib of anaplastic human cells, positive for the tyrosine kinase c-abl, induced a decrease in cell proliferation and invasiveness by reducing nuclear β-catenin and increasing β-catenin/E-cadherin binding to the plasma membrane. Imatinib attenuated TCF activity, which in turn reduced expression of its target gene *cyclin D1* leading to cell growth arrest (Rao et al., 2006). Treatment with Vandetanib of papillary TPC1 cells carrying a RET/PTC rearrangement also stabilized β-catenin in the plasma membrane, decreasing the expression of β-catenin target genes such as *c-myc* and *cyclin D1*, and decreasing cell growth and migration (Tartari et al., 2011). These results underscore the importance of Wnt pathway activation in thyroid cancer progression.

Interestingly, non-steroidal anti-inflammatory drugs such as Sundilac, which target the Wnt/β-catenin pathway, have been used in colon cancer treatment (Rice et al., 2003). Sundilac also reduces β-catenin expression, which is accompanied by a decrease in cell growth in human PTC cell lines overexpressing BRAF^{V600E} but not RET/PTC3 (Cho et al., 2010).

Finally, a conditionally replicative adenovirus harbouring the E1A and E1B expression under the control of TCF response elements has been reported. These constructs replicate specifically in cells with an active Wnt/β-catenin pathway. This therapeutic approach has been used in xenograft tumors in nude mice developed from several thyroid cancer cell lines with good results regarding tumor size reduction, and should be further developed in the future (Abbosh et al., 2007).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

It has become evident that the Wnt pathways are involved in thyroid cancer progression.

Wnt/β-catenin-independent pathways appear to act as tumor suppressors, because downregulation of Wnt5A, an activator of the non-canonical pathways, is needed to enhance the migratory and invasive capacity of thyroid tumor cells. The Wnt/β-catenin-dependent pathway, on the other hand, seems to be involved in the proliferation of normal thyroid cells, which is a highly controlled process.

In the earliest stages of progression of tumors such as FA or well-differentiated FTC and PTC, β-catenin remains mainly attached to the cell membrane, but begins to appear in the cytoplasm. In these early stages, tumors carrying RET/PTC rearrangements proliferate in a β-catenin-dependent way. Although in cell culture this enhanced proliferation correlates with nuclear accumulation of β-catenin and high expression of the *cyclin D1* and *c-Myc* genes, in tumor samples no such correlations were found and β-catenin was visible only in the cytoplasm. Further studies are needed to understand the correlation between cytoplasmic β-catenin, the expression of its cell cycle target genes, and the role of β-catenin in these tumors.

In poorly and undifferentiated carcinomas, β-catenin is found in the nuclei, and mutations in this gene or in other genes of the pathway such as *Axin1* induce the constitutive activation of the canonical Wnt pathway, which triggers an increase in proliferation.

In conclusion, β-catenin has a direct role in the proliferation of poorly and undifferentiated thyroid tumor cells, but more studies are needed to establish the role of β-catenin in earlier stages of thyroid tumor progression.

The use of animal models like APCMin or ΔNβ-catenin mice, which have a constitutively activated Wnt/β-catenin pathway, could be of great interest for better understanding the mechanism by which the Wnt pathway promotes tumor growth in thyroid cancer.

The Wnt/β-catenin pathway is involved in the transformation of a large number of tumors, and for this reason in the past few years several groups have been looking for specific inhibitors of this pathway. These inhibitors act through several mechanisms: increasing the stability of the destruction complex (Huang et al., 2009) in order to decrease cytoplasmic and nuclear β-catenin levels, or disrupting the interaction between β-catenin and the

transcription factors TCF/LEF (Lepourcelet et al., 2004) abolishing the transcription of cell cycle genes. The development of these inhibitors could contribute to the treatment of thyroid carcinomas, especially some anaplastic carcinomas, which show a clear activation of the Wnt/β-catenin pathway and have a poor prognosis due to their aggressiveness and the loss of differentiation markers.

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Thyroid cancer cell lines: critical models to study thyroid cancer biology and new therapeutic targets

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Thyroid cancer is the most common endocrine malignancy and the incidence is rising. Currently, there are no effective treatments for patients with advanced forms of thyroid cancer. Anaplastic thyroid represents the most severe form of the disease with 95% mortality at 6 months. It is therefore critical to better understand the mechanisms involved in thyroid cancer development and progression in order to develop more effective therapeutic strategies. Cell lines derived from thyroid tumors represent a critical tool to understand the oncogenic mechanisms driving thyroid cancer, as well as preclinical tools to study the efficacy of new therapies *in vitro* and *in vivo*. For thyroid cancer, the development of new therapies has been hampered by the lack of thyroid cancer cell lines in the widely used NCI-60 panel which has been used to screen over 100,000 anti-cancer drugs. In addition, the recent discovery that ~20 out of 40 existing thyroid cancer cell lines are either redundant or misidentified with cell lines of other tissue lineages has further hampered progress in the field. Of the available cell lines, 23 were identified as unique and presumably of thyroid origin based on the expression of thyroid-specific genes. Thus, there is a great need for validated thyroid cancer cell lines representing different stages of disease in addition to distinct oncogenic mutations. New, authenticated thyroid cancer cell lines are beginning to be developed, adding to the tools available to study genes and pathways important for thyroid cancer pathogenesis. In summary, the use of validated thyroid cancer cell lines that closely recapitulate disease is critical for the discovery of new drug targets and ultimately new therapies.

Keywords: thyroid cancer cell lines, cell line misidentification, authenticated thyroid cancer cell lines, ATC, PTC, FTC

INTRODUCTION

There are currently no effective therapies for patients with advanced thyroid cancer, which includes patients diagnosed with advanced papillary thyroid cancer (PTC) and anaplastic thyroid cancer (ATC; Pfister and Fagin, 2008; Smallridge et al., 2009). More than 1300 patients with thyroid cancer die each year from this disease and the incidence is increasing (Leenhardt et al., 2004; Davies and Welch, 2006; Xing, 2008; Enewold et al., 2009). ATC is one of the most aggressive human cancers with greater than 95% mortality at 6 months. Extrathyroidal invasion and metastasis are the most common causes of thyroid cancer-related death, and although much effort has been devoted to decipher the mechanisms involved in the progression of this cancer, little progress has been made in the development of new, effective therapies at this stage (Pfister and Fagin, 2008; Smallridge et al., 2009). Much attention has been devoted to the mitogen-activated protein kinase (MAPK) pathway as a therapeutic target due to the large percentage of activating mutations in this pathway (*BRAF*, *RAS*, *RET/PTC*; Gupta-Abramson et al., 2008), and although these studies are promising, additional therapeutic strategies are needed for the effective long-term treatment of advanced thyroid cancer patients (Pfister and Fagin, 2008).

Cell lines represent a critical tool to study mechanisms driving cancer initiation and progression, and remain an essential preclinical model to test new therapies. Although results using

cell lines do not always recapitulate the clinical response, cell lines nonetheless remain an essential tool to test pro-tumorigenic mechanisms and new therapies. Despite the importance of cell lines, recent studies have shown that 18–36% of cell lines are misidentified or cross-contaminated with other cell lines, thus compromising the interpretations of numerous published studies (Masters et al., 2001; Chatterjee, 2007; Lacroix, 2008; American Type Culture Collection Standards Development Organization Workgroup ASN-0002, 2010). Cross-contamination with the HeLa cell line is a well-known historical problem (MacLeod et al., 1999). More recently, cross-contamination of newly developed cell lines with older, established cell lines is becoming an increasingly recognized problem. Indeed, numerous misidentifications have been reported, some within the well-studied NCI-60 panel of cell lines (Lorenzi et al., 2009), in addition to cell lines thought to be of prostate (van Bokhoven et al., 2001a,b, 2003), breast (Rae et al., 2004, 2007; Liscovitch and Ravid, 2007), esophageal (Boonstra et al., 2007), adenoid cystic carcinoma (ACC; Phuchareon et al., 2009; Zhao et al., 2011), and head and neck squamous cell cancer (HNSCC) origin (Zhao et al., 2011).

MISIDENTIFICATION OF THYROID CANCER CELL LINES

In 2008, we reported the comprehensive characterization of a panel of 40 thyroid cancer cell lines (Scheweppe et al., 2008). Using genetic profiling, we showed that approximately 40% of these cell

lines were either cross-contaminated with other thyroid cancer cell lines, or misidentified with a cell lines from other tumor types. Specifically, 6 out of 40 cell lines were redundant (identical to other thyroid cancer cell lines), and 10 were misidentified with melanoma or colon cancer cell lines (Schweppe et al., 2008). For these studies, we used short tandem repeat (STR) profiling, which is the internationally approved method for determining cell line identity (Masters et al., 2001). Although STR profiling provides a powerful approach to determine genetic identity, it does not provide information on tissue of origin. Thus, we analyzed expression of the thyroid-specific transcription factors, Pax-8 and TTF-1 to further characterize the origin of these cell lines. For the misidentified cell lines of non-thyroid origin, expression of Pax-8 and TTF-1 were low to undetectable by quantitative real-time RT-PCR (qRT-PCR; unpublished observations). Of the remaining 23 unique cell lines that are likely of thyroid origin, all PTC and FTC cell lines expressed either Pax-8 and/or TTF-1, and about half of the ATC cell lines expressed one or both of these thyroid-specific transcription factors (Schweppe et al., 2008). While expression of Pax-8 and/or TTF-1 do not prove or disprove the origin of these cell lines, the presence of either Pax-8 or TTF-1 supports these cells being of thyroid origin. Critically, none of the unique thyroid cancer cell lines available at the time of this study have been genetically linked to their corresponding tumors, due to the lack of original patient tissue samples. Thus, it is difficult to determine when these misidentifications and cross-contaminations occurred. However, based on the timeline of establishment, it is likely that these cell lines were established by cross-contamination at the time of development (Schweppe et al., 2008). To add to the number of misidentified thyroid cancer cell lines, the ONCO-DG-1 “thyroid cancer” cell line was shown to be a misidentified with an ovarian cancer cell line, the FB2 “thyroid cancer” cell line was shown to be a derivative of the TPC1 cells, and the K1 cell line is likely a derivative of the GLAG66 thyroid cancer cell line (Ribeiro et al., 2008; Schweppe et al., 2008). Thus, it is clear that the development and characterization of new, authenticated thyroid cancer cell lines that are linked to their corresponding tissue samples is needed to complement studies using the existing panel of genetically unique cell lines to accurately study the mechanisms of thyroid cancer pathogenesis, and for the development of new therapeutic strategies.

NEW MODELS TO STUDY THYROID CANCER BIOLOGY

The development of new cancer cell lines is a challenging task with a low success rate, even for more aggressive tumors, and the establishment of cell lines of thyroid origin appears to be a particularly challenging task. Compared to other tumor types, the number of thyroid cancer cell lines is relatively small. The reasons for this low success rate are unclear, but could be partly due to the overall less aggressive nature of these tumors. Of the 784 cancer cell lines in the SANGER database¹, which maintains data for cancer cell lines that are widely available to the scientific community, only 5 of these are thyroid. Similarly, other major databases, including GlaskoSmithKline (GSK), which contains

genomic profiling data for >300 cancer cell lines², and the Broad-Novartis Cancer Cell Encyclopedia, which has data for 1000 cell lines³, also only contain a handful of thyroid cancer cell lines. While we identified 23 unique thyroid cancer cell lines, many of these are not available in major repositories. The American Type Culture Collection (ATCC) contains one thyroid cancer cell line of medullary thyroid cancer (MTC) origin, while the German Collection of Microorganisms and Cell Cultures (DSMZ) and European Collection of Cell Cultures (ECACC) contain a handful of genetically unique human thyroid cancer cell lines. Thus, the development of new thyroid cancer cell lines genetically linked to the original tissue sample is needed to improve our understanding of thyroid cancer biology, and once these cell lines are developed, these cell lines should be made available to the scientific community through a repository in order to maintain quality control.

Recently, Marlow et al. (2010) reported the development and characterization of four new ATC cell lines, THJ-11T, THJ-16T, THJ-21T, and THJ-29T. Of importance, STR profiling showed that the newly developed cell lines are genetically unique and match their corresponding tissue samples (Marlow et al., 2010). These THJ cell lines represent the first panel of thyroid cancer cell lines that has been genetically linked to their corresponding tissue of origin, providing a critical new tool to accurately study ATC biology. Interestingly, these cell lines exhibited similar patterns of chromosomal losses and gains compared to the original tumor tissue, as determined by array-based comparative genomic hybridization (CGH) analysis, suggesting that minimal genetic drift has occurred in culture (Marlow et al., 2010). The morphology of these cell lines was shown to be squamoid, spindle, giant, or a combination of these morphologies, similar to the original tumor samples (Marlow et al., 2010). Many of the common oncogenic alterations in ATC were also identified in these cell lines, including mutations in *BRAF*, *TP53*, *RB*, *RAS*, and *PI3KCA* (Marlow et al., 2010). Consistent with the ATC origin of these cell lines, rearrangements in *RET/PTC* and *PAX-8/PPAR γ* were not identified. Oncogenic proteins, including β -catenin, cyclin D1, survivin, and Bcl2 were also evaluated and are expressed at varying levels in the different cell lines, providing new tools to study common oncogenic signaling pathways in thyroid cancer (Marlow et al., 2010). Thyroid-specific gene expression was also evaluated by RT-PCR in this panel of cell lines. Thyroglobulin (Tg), sodium iodide symporter (NIS), and Pax-8 expression was detected in all four cell lines, TTF-1 expression was detected in three of these cell lines, while none of these cell lines expressed thyroid peroxidase (TPO; Marlow et al., 2010). Although TSH receptor (TSHR) expression was detected in one cell line, TSHR was not correctly localized to the cell membrane. All four cell lines were also shown to be tumorigenic in nude mice (Marlow et al., 2010). Thus, the development and characterization of these new ATC cell lines, which appear to closely represent their tumor counterparts, will provide valuable models to study the heterogeneity of ATC biology, and will be important tools to test the efficacy of new therapies using *in vivo* preclinical models.

²<http://array.nci.nih.gov>

³<http://www.broadinstitute.org/ccll/home>

Another set of new thyroid cancer cell lines was recently developed from follicular thyroid tumors derived from genetically engineered mice expressing a loss-of-function *Pten* allele and an oncogenic *Kras*, which results in constitutive activation of the phosphatidylinositol-OH kinase (PI3K) and MAPK pathways, a common event in advanced thyroid cancers (Miller et al., 2009; Dima et al., 2011). Unlike many immortalized cancer cell lines established from advanced tumors, these three mouse cell lines (T683, T691, and T826) were shown to have a relatively normal karyotype, with normal chromosome numbers, and only rearrangements in chromosome 4 for two of the cell lines (Dima et al., 2011). Common genetic alterations relevant to thyroid cancer were also tested. Two of the cell lines exhibited complete loss of the tumor suppressors, p16 and p19ARF, while p53 appeared to be functional (Dima et al., 2011). Consistent with a poorly differentiated phenotype, expression of genes important for thyroid differentiation and function, including *Foxe1*, *Nkx2-1*, *Pax-8*, *Duox1/2*, *Nis*, *Pds*, *TG*, *Tpo*, and *Tshr*, were absent (Dima et al., 2011). Due to constitutive activation of the PI3K and MAPK pathways in these cell lines, inhibition of these pathways with small molecule inhibitors was tested. As expected, inhibition of either pathway alone resulted in partial inhibition of growth, and dual inhibition of both the PI3K and MAPK pathways was more effective, similar to their results using primary cells derived from these tumors (Miller et al., 2009; Dima et al., 2011). Since PI3K can mediate activation of the glycolytic pathway in tumors, lactate production and glycolytic metabolism was also evaluated. Compared to normal thyrocytes, lactate production was higher and cell growth was blocked by a glycolysis inhibitor, suggesting that these FTC-derived cell lines have likely switched to a glycolytic mode, and that inhibition of glycolysis may be a potential new therapy for FTC (Dima et al., 2011). Finally, when implanted into immunocompetent mice, all three cell lines were shown to grow tumors and these mice developed lung metastases (Dima et al., 2011). Thus, these new cell lines represent important new tools to study the mechanisms of PI3K and MAPK signaling, and provide a new model to study FTC tumor growth and metastasis in an *in vivo* immunocompetent preclinical model (Dima et al., 2011).

DEVELOPMENT OF NEW THERAPIES FOR THYROID CANCER

The recent discovery of misidentified thyroid cancer cell lines has certainly hampered the progress in the field (Ringel, 2008; Schweppe et al., 2008). These misidentified cell lines have been widely used in over 300 publications in the last 20 years, and as recently reviewed by Kojic et al. (2011), these misidentified thyroid cancer cell lines, especially DRO90, ARO81, and NPA87, have been used in a significant number of preclinical studies to test new targeted therapies for ATC. Thus, numerous studies using these misidentified cell lines require reinterpretation and validation. The development of new therapies for thyroid cancer patients has also been slowed by the lack of thyroid cancer cell lines in the NCI-60 panel, which has been used to screen >100,000 anti-cancer agents over the last ~20 years, and has been widely used to study global gene expression patterns and genomic alterations (Shoemaker, 2006). Despite these problems, as outlined below, progress has been made testing new therapies in validated thyroid

cancer cell lines, and overall thyroid cancer cell lines have made major contributions to the understanding of thyroid cancer biology and the identification of new candidate targets for therapy (Kojic et al., 2011).

Many of the oncogenic events contributing to thyroid cancer pathogenesis have been identified. Genetic alterations in components of the MAPK pathway, including *BRAF*, *RAS*, and *RET/PTC* are common in PTC, while mutations in *RAS* and *PPAR γ /Pax-8* rearrangements are prevalent in FTC (Knauf and Fagin, 2009; Saji and Ringel, 2010; Xing, 2010; Carlomagno and Santoro, 2011). For ATC, mutations in *BRAF* and *RAS* are common, and many of these cancers harbor genetic alterations in *PI3KCA* and *TP53* along with activating mutations in the MAPK pathway (Knauf and Fagin, 2009; Saji and Ringel, 2010; Xing, 2010; Carlomagno and Santoro, 2011). Recently, a high proportion (~80%) of metastatic thyroid tumors was shown to exhibit dual activation of the MAPK and PI3K pathways due to oncogenic mutations in *BRAF* and *PIK3CA* or *BRAF* and *AKT1* (Ricarte-Filho et al., 2009). Thus, the development of new therapeutic strategies targeting these pathways is of great interest.

Thyroid cancer cell lines provide an important source to better understand oncogenic signaling mechanisms and to develop new and improved therapies. Of importance, many of the oncogenic alterations found in thyroid cancer, including mutations in *BRAF*, *RAS*, *PI3KCA*, and *RET/PTC1* are represented in currently available thyroid cancer cell lines (Schweppe et al., 2008). While early studies used many of the misidentified cell lines to test agents targeting the MAPK and PI3K signaling pathways, subsequent studies using validated thyroid cancer cell lines have confirmed these results (Xing, 2009). Similar to studies in melanoma, thyroid cancer cell lines harboring a *BRAF V600E* mutation are in general more sensitive to treatment with MKK1/2 and BRAF-specific inhibitors (Solit et al., 2006; Leboeuf et al., 2008; Liu et al., 2009, 2011a; Schweppe et al., 2009a; Salerno et al., 2010; Nucera et al., 2011). Despite the presence of an activating BRAF mutation, clinical studies are indicating that the response of thyroid cancer patients to MAPK-directed therapies is not as promising as melanoma patients. These results are consistent with preclinical studies using cell lines, where Montero-Conde et al. (2011) have shown BRAF-mutant thyroid cancer cell lines are less sensitive to selective BRAF V600E inhibition compared to BRAF-mutant melanoma cell lines. The reason(s) for this differing sensitivity are unclear, but may be due to compensatory upregulation of other pathways, including ErbB3 (Montero-Conde et al., 2011). Alternatively, EGFR signaling was recently shown to promote survival in response to BRAF or MEK1/2 inhibition in BRAF-mutant thyroid and colon cancer cells, but not in melanoma (Prahallad et al., 2012). Although further studies are needed in thyroid cancer, these results suggest that combined inhibition of the MAPK pathway and ErbB2 or EGFR may be beneficial in BRAF-mutant thyroid tumors.

Activation of the PI3K pathway represents another major oncogenic pathway in thyroid cancer. Recent studies targeting the PI3K pathway have shown that thyroid cancer cells with activation of the PI3K pathway are preferentially sensitive to inhibitors of this pathway (Liu et al., 2009, 2011a). Despite these promising results, it is not clear whether inhibition of the PI3K pathway alone will

have sufficient activity in advanced thyroid cancers as single agent therapies. Indeed, recent studies have shown that dual targeting of the MAPK and PI3K pathways may represent a more promising strategy for thyroid tumors that harbor genetic alterations in both pathways (Jin et al., 2009; Liu et al., 2010, 2011b). These studies are consistent with the work from Dr. Di Cristafano's laboratory (discussed above) showing genetic activation of both the MAPK and PI3K pathway is necessary for transformation of the thyroid gland, and that dual inhibition of these pathways is more effective than inhibition of either pathway alone (Miller et al., 2009; Dima et al., 2011). Along with the discovery of co-existing mutations of *BRAF V600E* with *AKT1* or *PIK3CA* mutations in metastatic thyroid lesions (Ricarte-Filho et al., 2009), these studies indicate that dual targeting of the MAPK and PI3K pathways represent a promising therapeutic strategy for patients with advanced thyroid cancer.

Another promising therapeutic strategy for PTC and ATC is targeting tyrosine kinase signaling with tyrosine kinase inhibitors (TKIs). Of these, VEGF family members and its receptors have been shown to play an important role in thyroid cancer, independent of oncogene mutational status (Castellone et al., 2008; O'Neill et al., 2010). Several studies have shown that VEGF and VEGFR2 are overexpressed in thyroid tumors, and that VEGF overexpression is associated with decreased disease-free survival and a poor prognosis. Although initial reports used misidentified cell lines to test the role of VEGFR in PTC and ATC (Kim et al., 2005), recent preclinical studies have shown that treatment with vandetanib (ZD6474), a multi-kinase inhibitor of VEGFR, EGFR, and RET, inhibits proliferation of ATC cells *in vitro*, likely via inhibition of the EGFR receptor, and blocks tumor growth in an *in vivo* orthotopic ATC model, primarily through anti-angiogenic mechanisms which are likely mediated by VEGFR2 (Gule et al., 2011). In support of these preclinical studies, several phase II clinical trials with anti-VEGFR multi-kinase inhibitors are underway, including vandetanib, sunitinib, axitinib, sorafenib, motesanib, and XL-184, with variable, but encouraging results (Castellone et al., 2008; Perez et al., 2011).

The Src–focal adhesion kinase (FAK) tyrosine kinase pathway is another emerging therapeutic target for thyroid cancer (Schweppke et al., 2009b). Src and FAK are multifunctional non-receptor tyrosine kinases that are key regulators of growth, survival, migration, and invasion (Kopetz et al., 2007; Schwock et al., 2010). In one previous study, FAK protein was shown to be overexpressed in a subset of PTC and ATC, but the phosphorylation status of FAK was not examined (Kim et al., 2004). We were the first to

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show that FAK is phosphorylated in a subset of PTC patient tumor samples (Schweppke et al., 2009b). We further showed that FAK is phosphorylated in a panel of validated thyroid cancer cells, and that the growth and invasion of cells with high phospho-FAK are sensitive to treatment with the Src inhibitor, saracatinib (AZD0530; Schweppke et al., 2009b). Inhibition of growth and invasion was independent of oncogenic mutations in the MAPK pathway, suggesting that the FAK-Src pathway represents another major pro-tumorigenic signaling pathway in thyroid cancer, independent of MAPK signaling. Clinical trials are underway testing Src inhibitors in solid tumors, including BMS-354,825 (dasatinib; Bristol-Myers Squibb), bosutinib (SKI-606, Wyeth; Quintas-Cardama et al., 2007), and the more selective Src inhibitor, AZD0530 (saracatinib; AstraZeneca; Hennequin et al., 2006; Santini et al., 2010). Recently, FAK inhibitors have been developed, and two ATP-dependent small molecule inhibitors, including NVP-TAE-226 and the more selective PF-562,271, have entered clinical trials (Halder et al., 2007; Roberts et al., 2008; Siu et al., 2008; Schwock et al., 2010). Thus, future studies testing the efficacy of Src and FAK inhibitors in advanced thyroid cancer will be of great interest.

CONCLUDING REMARKS

Progress is being made in the development and characterization of new thyroid cancer cell lines. The majority of currently available cell lines represent PTC and ATC, while FTC-derived cell lines remain underrepresented. Importantly, while the major oncogenic mutations found in thyroid tumors are represented in many of the available cell lines (*BRAF*, *RAS*, *PI3KCA*, *RET/PTC1*), some of the less common genetic alterations, including *AKT1*, which is likely important in metastatic thyroid cancer (Ricarte-Filho et al., 2009), specific RET/PTC isoforms, which play distinct roles in PTC pathogenesis, and PPAR γ /Pax-8 rearrangements, which are important in FTC, are not represented in the current panel of cell lines (Schweppke et al., 2008). For further translational relevance, studies with permanent cell lines should be complemented with studies using human tissue samples, and when possible, primary culture models, which may better recapitulate the original tumor. In conclusion, the continued development and characterization of new cell line models that are genetically linked to the original patient tissue samples is critical to further understand the oncogenic properties of thyroid cancer cells, identify novel targets for therapy, and to translate these findings to the clinic for patients with advanced thyroid cancer.

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Thyroid cancer cell lines: an overview

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Human thyroid cancer cell lines are the most used models for thyroid cancer studies. They must be used with detailed knowledge of their characteristics. These *in vitro* cell lines originate from differentiated and dedifferentiated *in vivo* human thyroid tumors. However, it has been shown that mRNA expression profiles of these cell lines were closer to dedifferentiated *in vivo* thyroid tumors (anaplastic thyroid carcinoma, ATC) than to differentiated ones. Here an overview of the knowledge of these models was made. The mutational status of six human thyroid cancer cell lines (WRO, FTC133, BCPAP, TPC1, K1, and 8505C) was in line with previously reported findings for 10 genes frequently mutated in thyroid cancer. However, the presence of a BRAF mutation (T1799A: V600E) in WRO questions the use of this cell line as a model for follicular thyroid carcinoma (FTC). Next, to investigate the biological meaning of the modulated mRNAs in these cells, a pathway analysis on previously obtained mRNA profiles was performed on five cell lines. In five cell lines, the MHC class II pathway was down-regulated and in four of them, ribosome biosynthesis and translation pathways were up-regulated. mRNA expression profiles of the cell lines were also compared to those of the different types of thyroid cancers. Three datasets originating from different microarray platforms and derived from distinct laboratories were used. This meta-analysis showed a significant higher correlation between the profiles of the thyroid cancer cell lines and ATC, than to differentiated thyroid tumors (i.e., PTC or FTC) specifically for DNA replication. This already observed higher correlation was obtained here with an increased number of *in vivo* tumors and using different platforms. In summary, this would suggest that some papillary thyroid carcinoma or follicular thyroid carcinoma (PTC or FTC) cell lines (i.e., TPC-1) might have partially lost their original DNA synthesis/replication regulation mechanisms during their *in vitro* cell adaptation/evolution.

Keywords: cell line, thyroid, cancer, mutation, WRO, FTC133, TPC1, BCPAP

INTRODUCTION

The experimental study of human cancers uses *in vitro* and *in vivo* models. Among the various possible experimental models, human cancer cell lines are frequently used (van Staveren et al., 2009). They have retained hallmarks of cancer cells; they are pure, genetically identical, easily propagated and can be genetically manipulated. A cell line originates from a tissue and is obtained by selection of the most rapidly proliferating and resistant cells in monolayer during passages.

Results obtained on a cell line are sometimes directly extrapolated for *in vivo* cancers which produced this cell line (Yeung et al., 2007; Wang et al., 2008). However, the representativity of the cancer cell line may be distorted by a cross contamination of one cell line by another (Ribeiro et al., 2008; Schweppe et al., 2008), an *in vitro* evolution of the cell line (van Staveren et al., 2009), a strong genomic instability due to the number of passages or a risk of infection (Harlin and Gajewski, 2008). Thus, a systematic verification of the status of these cell lines is important (van Staveren et al., 2007; Ribeiro et al., 2008; Schweppe et al., 2008).

Thyroid cancer is the most frequent endocrine cancer (Kondo et al., 2006; Sipos and Mazzaferri, 2010). There are various types

of thyroid carcinomas, the carcinomas from thyrocyte are largely the most frequent: papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC) anaplastic thyroid carcinoma (ATC) and an intermediate form between PTC/FTC and ATC, the poorly differentiated thyroid cancer. Each type is characterized by a set of mutations leading to increased cellular proliferation and dedifferentiation (Catalano et al., 2010).

PTC is the most frequent type of human thyroid carcinoma (Kondo et al., 2006). The genetic alterations most often found in PTC are BRAF point mutations, accounting for 40–60% of the cases, and RET/PTC rearrangements which are present in about 20% of the cases. The most frequent BRAF mutation occurs in the serine/threonine kinase domain (V600E) and leads to the constitutive kinase activity of the protein (Xing, 2010). This mutation could be a key mutation for the treatment and the diagnosis of the most aggressive PTC (Nucera et al., 2010; Xing, 2010). RET/PTC rearrangements are the result of a fusion between the 3' end of a receptor of the tyrosine kinase family (RET) and the 5' end of a gene constitutively expressed in thyrocytes. The most frequent rearrangements of this type are RET/PTC1 and RET/PTC3. The fusion results in the constitutive activation of the truncated

tyrosine kinase portion of RET by autophosphorylation due to the dimerization domain of the heterologous gene (Catalano et al., 2010).

The genetic changes most often found in FTC are RAS point mutations (approximately 45% of the cases) and PAX8/PPAR γ rearrangements (approximately 35% of the cases). Mutations of the RAS gene activate the mitogenic MAPK and the PI3K pathways. PAX8/PPAR γ rearrangements are the result of a fusion between the 5' end of the PAX8 transcription factor and the 3' end of PPAR γ , a member of the nuclear hormone receptor superfamily, constitutively expressed in thyrocytes. Different rearrangements between the 2 genes have been described, and sometimes reported within a single tumor (Lacroix et al., 2004). PAX8/PPAR γ chimeric proteins have a dominant negative effect on the wild-type PPAR γ . In addition to these, alterations in the tumor suppressor gene PTEN (10% of the cases) and the PI3KCA oncogenes (10% of the cases) have been described in this tumor type.

ATC is the most dedifferentiated, aggressive thyroid cancer (Kondo et al., 2006). The genetic alterations most often observed are the ones already described in PTC and FTC with the exception of RET/PTC and PAX8/PPAR γ rearrangements, but in addition also include mutations in TP53 and β -catenin genes.

For each type of human thyroid carcinoma, derived cell lines have been generated. In this study, we used six different human cancer cell lines derived from different types of thyroid cancers. The WRO (Estour et al., 1989) and FTC133 (Goretzki et al., 1990) cell lines are derived from FTC; the BCPAP (Fabien et al., 1994) commonly known as a PTC cell line is derived from a poorly differentiated PTC; TPC1 (Tanaka et al., 1987) and K1 (Challeton et al., 1997) cell lines are derived, respectively, from PTC and from a metastasis of a well-differentiated PTC (Ribeiro et al., 2008); the 8505C (Ito et al., 1993) cell line from ATC. These six cell lines are among those mostly used for thyroid cancer research. The mutational status of each of these cell lines was investigated and compared to the literature. Next, pathways were further investigated by using our previously obtained data on mRNA expression patterns of these cell lines that had been compared to primary cultured normal human thyrocytes (van Staveren et al., 2007). Previously we showed that mRNA expression profiles from different human thyroid tumor cell lines, including cell lines of the present study, evolved *in vitro* into similar phenotypes with mRNA expression profiles closer to undifferentiated *in vivo* thyroid tumors (ATC) than to differentiated thyroid cancers (PTC and FTC) (van Staveren et al., 2007). To investigate this further, a correlation analysis between mRNA expression profiles from cell lines and mRNA expression profiles from each *in vivo* thyroid tumor type was performed by using three datasets generated in two different laboratories that were derived from different microarray platforms.

MATERIALS AND METHODS

CELL CULTURE

Cell lines were obtained from various laboratories. The WRO, FTC133, BCPAP, and 8505C cell lines were received from Prof. G. Brabant (Medizinische Hochschule, Hannover, Germany);

the TPC1 cell line from Prof. M. Mareel (University of Ghent, Belgium); the K1 cell line from Dr. Zaruhi Poghosyan (Cardiff University, School of Medicine, Cardiff, UK). The BCPAP, TPC1 and 8505C cell lines were cultured in RPMI 1640 (w L-glutamate) completed by foetal bovine serum (10%), penicillin/streptomycin (2%) and amphotericine B (1%); the FTC133, WRO and K1 cell lines were cultured in Dulbecco's Modified Eagle Medium, Nutrient mixture F-12 (1:1, by volume) completed by foetal bovine serum (10%), penicillin/streptomycin (2%), amphotericine B (1%). All culture reagents were purchased from Gibco (Paisley, UK).

RNA EXTRACTION

After removal of the culture medium, 2 ml of TRIzol Reagent (Ambion, Austin, USA) was added to the cells grown in 10 cm dishes. Total RNA was extracted according to the manufacturer's instructions, followed by purification on miRNeasy columns (QIAGEN, Hilden, Germany). RNA was spectrophotometrically quantified, and its integrity was verified by automated gel electrophoresis (Experion, Bio-Rad, Hercules, USA).

RT-PCR

After a DNase treatment with DNase I Amplification Grade kit (Invitrogen, Carlsbad, USA), 1 μ g of total RNA was used for reverse transcription using hexamers [(3.6 μ g/ μ l) (Roche, Basel, Switzerland)] and reverse transcriptase (Superscript II RNase H Reverse Transcriptase kit, Invitrogen). The PCR reactions were performed with the recombinant Taq DNA polymerase kit (Invitrogen). Each PCR reaction was performed in the presence of 5 μ l 10X PCR buffer, 1.5 μ l MgCl₂ (50 mM), 1 μ l dNTP mix (10 mM, Invitrogen), 1 μ l of the forward and the reverse primer (10 μ M each), 0.4 μ l Taq DNA polymerase (5U/ μ l), 2 μ l DNA from the RT reaction and 38.1 μ l of water. Primers and PCR conditions are detailed in **Table 1**.

SEQUENCING

PCR products were purified with the QIAquick PCR purification kit (QIAGEN) according to the manufacturer's instructions. Sequencing was performed with the BigDye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) with the sequencer ABI PRISM 3130 (Applied Biosystems) and the genetic analysis program 3130-XI. Sequences were analyzed with BLAST (Basic Local Alignment Search Tool program) from NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

PRE-PROCESSING OF THE DIFFERENT DATASETS

A merged file was created which contained three different datasets derived from three different microarray platforms. The first dataset consisted of five cell lines hybridized on IRIBHM custom slides, using primary cultured normal thyrocytes as a reference (www.ulb.ac.be/medecine/iribhm/microarray/data/) (van Staveren et al., 2007). The second dataset was composed of 11 ATC, 49 PTC, and 45 normal thyroid tissues hybridized on the Affymetrix HG U133 Plus 2.0 platform (GSE33630). The third dataset contained 4 ATC, 51 PTC, 13 FTC, and 4 normal thyroid tissues hybridized on the Affymetrix HG U133A platform (Giordano et al., 2005) (GSE27155). Each platform has been validated in previous

Table 1 | Targeted exons, sequences of the primers used for PCR amplification, amplicon length and PCR conditions for each investigated gene.

Gene	Targeted exons(s)	Forward primer (5'→3')	Reverse primer (5'→3')	Size (bp)	PCR conditions
BRAF	14 and 15	GCACAGGGCATGGATTACTT	GATGACTTCTGGTGCACATCC	194	Std but Tm at 55°C
NRAS	3	CGCACTGACAATCCAGCTAA	TCGCTTAATCTGCTCCCTGT	255	Std
HRAS	3	GGAAGCAGGTGGTCATTGAT	ACGTCACTCGAGTCCTTCAC	204	Std
KRAS	3	AGAGAGGCCTGCTGAAAATG	TTGACCTGCTGTGTCGAGAA	200	Std
TP53-1	2, 3, 4, 5	GTGACACGCTCCCTGGAT	ACACGCAAATTCCTTCAC	658	Std
TP53-2	5, 6, 7	CCCTTCCCAGAAAACCTACC	AGCTGTTCCGTCAGTAGA	518	Std
TP53-3	6, 7, 8, 9, 10, 11	GCTGCTCAGATAGCGATGGT	GTGGGAGGCTGTCAGTGG	660	Std
PI3KCA-1	9, 10, 11	TGACTGGTTCAGCAGTGTGG	GGCCAATTTTACCAAGCA	341	Std
PI3KCA-2	20, 21	TTTGACACAGGATTCTTAATAGTGA	GGTCTTGCTGCTGAGAGT	418	Std but Tm at 55°C
PAX8/PPAR γ	Pax8: 8, 9, 10 PPAR γ : 3 8, 9 8, 10 8, 9	GCAACCTCTGACTCACCAG (PAX8)	CATTACGGAGAGATCCACGG (PPAR γ)	407 305 217 108	Std
RET/PTC1	RET:12, 13 H4: 1	GGCACTGCAGGAGGAGAAC (H4)	GATGACATGTGGTGGTTGA (RET)	277	Std
RET/PTC3	RET:12, 13 ELE1: 7	AAGCAAACCTGCCAGTGG (ELE1)	TGCTTCAGGACAGTTGAAC (RET)	240	Std but with 30 cycles
PTEN-1	4, 5, 6	GACATTATGACACCGCCAAA	CGCCACTGAACATTGGAATA	405	Std
PTEN-2	6, 7, 8, 9	GCTACCTGTTAAGAATCATCTGGA	TGACGGCTCTACTGTTT	530	Std but Tm at 48°C
PBGD	11, 12, and 13	AAGGACCAGGACATCTTGGAA	AACTGTGGGTACCTCAGG	266	Std but Tm at 55°C

Std: Standard conditions: 94°C for 5 min followed by 35 cycles consisting of incubations at 94°C for 30 s, 60°C (Tm) for 1 min and 72°C for 1 min. At the end of cycles the PCR mixtures were incubated at 72°C for 10 min. PBGD gene was used as a positive control for amplification. For each PCR, a negative control (the amplification mix without DNA) was used to detect possible contamination.

studies [Giordano et al., 2005; van Staveren et al., 2006; Hébrant et al., 2012; Dom et al. (submitted)]. For both independent Affymetrix platforms, mRNA expression profiles of normal tissues were pooled as a reference and an mRNA expression ratio between each tumor and the normal reference was calculated for each gene. For the comparison of cell lines with *in vivo* thyroid tumors, only genes were included that was expressed in both cell lines and primary thyrocytes. Finally, the merged file contained the mRNA expressions of 2392 genes, expressed in log₂, present on the three different microarray platforms.

DAVID PATHWAY ANALYSIS

In order to investigate the biological meaning of the modulated mRNAs, Database for Annotation, Visualization and Integrated Discovery (DAVID pathway analysis) (Huang da et al., 2009) was used. For the cell line analyses, only genes showing a regulation of at least |0.58| in log₂, i.e., 1.5-fold or more were included. To calculate the background, all the genes present on the IRIBHM custom slides were used. For the comparison between ATC and cell lines, only significant commonly regulated genes were considered. The background was calculated using all the genes present in the merged file. For both analyses, a pathway was selected when its False Discovery Rate (FDR) was <5%. The analysis was performed separately for up-regulated and down-regulated genes.

CORRELATION OF mRNA EXPRESSION BETWEEN *in vitro* HUMAN THYROID CANCER CELL LINES AND *in vivo* HUMAN THYROID TUMORS

Spearman's correlation coefficients were computed using R (version 2.14.1). Based on the merged file, correlation coefficients between gene expression profiles of ATC and thyroid cancer cell lines were compared to correlation coefficients between gene expression profiles of PTC or FTC and thyroid cancer cell lines, using a Mann-Whitney *U*-test. This assessed whether ATC and cell lines had more similar mRNA expression profiles than FTC or PTC and cell lines. Comparisons of those coefficients were made between samples hybridized on the same microarray platform. 2D-multidimensional scaling was plotted based on those Spearman correlation coefficients using the R function isoMDS from the package MASS v.7.3–18.

ANALYSIS OF THE GENES COMMONLY AND OPPositely MODULATED IN ATC *in vivo* AND CANCER CELL LINES

Scores and *q*-values were computed based on a slightly modified version of Rank Products (Breitling et al., 2004) to find genes regulated in ATC compared to normal tissues and in cell lines compared to primary cultured normal thyrocytes. Rank Products is a non-parametric method allowing to study differential expression and to integrate the information of different datasets regardless of the potential technical differences, e.g., from different laboratories or different microarray platforms. It has been shown to outperform SAM (Zang et al., 2007) and is

more powerful with fewer samples than *t*-test based statistics. The genes with a *q*-value ≤ 0.05 were selected from ATC and cell lines. Genes that were regulated in the same direction in ATC and cell lines were kept and are shown in a heatmap using R library: gplots v.2.10.1 (<http://cran.r-project.org/web/packages/gplots/index.html>). Similarly, genes that had significant regulations in opposite directions between ATC and cell lines were further analyzed.

RESULTS

MUTATIONAL STATUS

The mutational status was investigated for known thyroid oncogenes and tumor suppressor genes in six human thyroid cancer cell lines. For each cell line, the presence of point mutations in *BRAF*, *NRAS*, *HRAS*, *KRAS*, *TP53*, *PI3KCA*, *PTEN* genes was explored as well as the presence of RET/PTC1, RET/PTC3, and PAX8/PPAR γ rearrangements. Therefore, primers were designed to target the point mutations and the rearrangements most frequently found in thyroid carcinomas (Kondo et al., 2006) and each mutation was verified by sequencing both DNA strands. The results are summarized in **Table 2**. Neither *NRAS*, *HRAS*, *KRAS* point mutations nor RET/PTC3 or PAX8/PPAR γ gene rearrangements were detected in any of the six cell lines. As reported earlier, FTC133 cells showed *PTEN* and *TP53* mutations, BCPAP and 8505C cells showed *BRAF* and *TP53* mutations, and TPC1 cells harbored the RET/PTC1 gene rearrangement. WRO cells presented the heterozygous *BRAF* T1799A (V600E) mutation and K1 the heterozygous *PIK3CA* G1624A (E542K) mutation, in addition to the *BRAF* mutation (**Figure 1**).

PATHWAY ANALYSIS OF THE CELL LINES

To define the modulated pathways in thyroid cancer cell lines, the genes from previously published mRNA expression profiles from five thyroid cancer cell lines (FTC133, BCPAP, 8505C, TPC1, WRO) were further analysed (van Staveren et al., 2007). These data reported the differential mRNA expression between each cell line and a pool of human primary cultured normal thyrocytes. DAVID pathway analyses were performed on each individual cell line (Supplementary information A). Four of the five cell lines, i.e., WRO, BCPAP, TPC1, and 8505C, showed commonly regulated pathways. An upregulation of ribosome biosynthesis and translation was detected in these four cell lines, and in addition, expression of genes involved in the cell cycle was increased in WRO and BCPAP cells. Expression of genes involved in DNA replication was up-regulated in both BCPAP and 8505C cells.

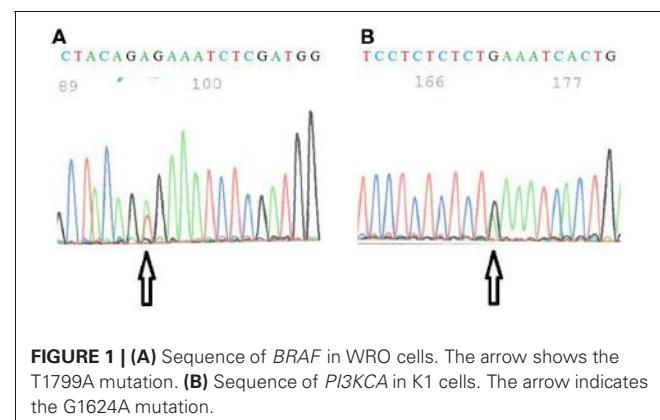


FIGURE 1 | (A) Sequence of *BRAF* in WRO cells. The arrow shows the T1799A mutation. **(B)** Sequence of *PI3KCA* in K1 cells. The arrow indicates the G1624A mutation.

Table 2 | Mutational status of six human thyroid cancer cell lines derived from three types of thyroid carcinomas.

Cell lines	BRAF	NRAS	HRAS	KRAS	PI3KCA	PTEN	TP53	RET/PTC1	RET/PTC3	PAX8/PPARγ
WRO (FTC)	GTG-> GAG V600E*	wt	wt	wt	wt	wt	wt	–	–	–
FTC133 (FTC)	wt	wt	wt	wt	wt	CGA->TGA R130STOP	CGT->CAT R273H	–	–	–
B-CPAP (PTC)	GTG-> GAG V600E	wt	wt	wt	wt	wt	GAC->TAC D259Y	–	–	–
TPC1 (PTC)	wt	wt	wt	wt	wt	wt	wt	+	–	–
K1 (PTC)	GTG-> GAG V600E*	wt	wt	wt	GAA->AAA E542K*	wt	CGA->CGC R213R	–	–	–
8505c (ATC)	GTG-> GAG V600E	wt	wt	wt	wt	wt	CGG->GGG R248G	–	–	–

Differences between these results and the published data are indicated in bold. These results were compared to previously published data for WRO (Namba et al., 2003; Xu et al., 2003; Garcia-Rostan et al., 2005; Paloma et al., 2006; Schweppe et al., 2008), FTC133 (Ricarte-Filho et al., 2009), B-CPAP (Garcia-Rostan et al., 2005; Meireles et al., 2007; Ricarte-Filho et al., 2009), TPC1 (Meireles et al., 2007; Ricarte-Filho et al., 2009), K1 (Garcia-Rostan et al., 2005; Meireles et al., 2007; Ricarte-Filho et al., 2009) and 8505c (Garcia-Rostan et al., 2005; Meireles et al., 2007; Ricarte-Filho et al., 2009; Nucera et al., 2010) cell lines. *, heterozygous mutation; wt, wild-type genotype (no mutation); +, presence of the gene rearrangement; –, absence of the gene rearrangement.

Furthermore, in all cell lines, expression of genes involved in MHC class II biosynthesis was down-regulated and in four cell lines (not in 8505C) the expression of genes involved in negative regulation of cell death/apoptosis was also down-regulated. In both 8505C and TPC1 cells, expression of genes involved in the immune response was decreased and for the latter, a decrease in the protease inhibitor pathway was also observed. In contrast, the FTC133 cell line showed some differences in the regulated pathways. Although the downregulation of the MHC class II and the negative regulation of cell death/apoptosis were present, no increase of expression of genes involved in ribosome biosynthesis and translation were observed. Instead, FTC133 cells showed a strong upregulation of global RNA processing.

CORRELATIONS BETWEEN mRNA EXPRESSION PROFILES OF *in vitro* THYROID CANCER CELL LINES AND mRNA EXPRESSION PROFILES OF *in vivo* THYROID TUMORS

Two different datasets of mRNA expression profiles from *in vivo* human thyroid tumors were correlated to the mRNA profiles of the WRO, FTC133, BCPAP, TPC1, and 8505C cell lines. One dataset was composed of 11 ATC and 49 PTC hybridized on Affymetrix HG U133 Plus 2.0 arrays. The other dataset consisted of 4 ATC, 13 FTC, and 51 PTC hybridized on Affymetrix HG U133A arrays. For both independent datasets, the Spearman correlation coefficient was the highest between cell lines and ATC compared to the cell lines and PTC or FTC (Figures 2A,B). Furthermore, there was a significant difference between the correlations of ATC versus cell lines compared to those calculated with the PTC or FTC profiles (Figures 2A,B).

These results are in line with previous results which showed by 2D-multidimensional scaling analysis the highest similarity between cell lines and ATC, compared to cell lines with PTC or FTC (van Staveren et al., 2007). Thus, this already observed relation could be confirmed in the present study using more tumor samples that had been hybridized on different microarray platforms. As shown in Figure 2C, although a platform effect was visible, in both independent datasets ATC were closer to cell lines.

ANALYSIS OF THE GENES COMMONLY AND OPPositely MODULATED IN ATC *in vivo* AND CANCER CELL LINES

To further investigate the similarity between ATC and cell lines, their mRNA expression profiles, obtained on different microarray platforms, were compared. Rank Products and SAM were used to find genes significantly regulated in the same direction or in the opposite direction comparing ATC with cell lines. Sixty-one genes were found to be up-regulated in both ATC and cell lines and eighty-four genes were found to be commonly down-regulated (Figure 3). Eighteen genes were regulated in an opposite direction between ATC and cell lines.

To gain more insight into those regulations, a DAVID pathway analysis was performed (Supplementary information B). Some common up-regulated genes in ATC and cell lines are involved in DNA replication and DNA metabolic processes, e.g., *TOP2A*, *MCM7*, *CHAF1A*, or *HMGB2*. Most of the 18 genes that were regulated in an opposite direction were down-regulated in cell lines but up-regulated in ATC. They are involved in MHC class

II pathway or in cell adhesion, e.g., *HLA-DPB1*, *HLA-DQB1*, *HLA-DRA*, *ICAM1*, *CCL2*, or *COL6A3*.

DISCUSSION

In this work, the aim was to further investigate the characteristics for different human thyroid cancer cell lines. First, genetic alterations in these cell lines were confirmed. Second, regulated pathways in these cell lines were further explored. Third, correlations, based on gene expressions, between *in vitro* human thyroid cell lines and *in vivo* human thyroid tumors were analyzed. Finally, the similarity between cell lines and ATC was further explored on the gene level.

MUTATIONAL STATUS

We have investigated the mutational status of 10 commonly mutated genes in thyroid cancer by using six different cell lines. STR analyses on these cell lines (van Staveren et al., 2007 and Supplementary information C) showed that they were identical to those published by Schweppe and coauthors (Schweppe et al., 2008). No differences with reported literature data were observed for the mutational status of these genes in BCPAP, FTC133, TPC1, and 8505C cell lines. However, a difference could be observed for the status of *BRAF* in WRO and *PI3KCA* in K1 cells.

For WRO cells described as a FTC cell line, the *BRAF* T1799A (V600E) mutation was detected although these cells were originally described as *BRAF* wild-type (Namba et al., 2003; Xu et al., 2003) and are still frequently used as such today (Palona et al., 2006; Liu et al., 2007; Burrows et al., 2010; Hou et al., 2010). The presence of a *BRAF* mutation in these cells was nevertheless reported by Schweppe and coworkers (Schweppe et al., 2008) and they demonstrated that the cell line was not contaminated by another cell line harboring this mutation. It has been suggested that two different WRO cell lines were distributed. The WRO cells from Schweppe's laboratory has the same DNA profile (STR analysis) than ours (van Staveren et al., 2007; Schweppe et al., 2008), but is different from that of the European Collection for Cell Culture. The presence of this mutation in WRO cells was unexpected because this cell line has been derived from a FTC, and the V600E *BRAF* mutation is described as a specific characteristic for PTC. This might suggest that, in spite of its initial diagnosis of follicular cancer, the tumor at the origin of the creation of the WRO cell line would have rather been a papillary type. The presence of a mixture of *BRAF* mutated and non-mutated cells in the original tumor sample cannot be excluded. However, it may also be proposed that this cell line acquired this mutation *in vitro*, during its successive divisions in an artificial environment of selection (van Staveren et al., 2007, 2009). These observations question the representativity and the utility of this cell line as a model for human FTC and show the necessity of a systematic check of the cell lines. Furthermore, the V600E *BRAF* mutation is not specific of thyroid cancer. The origin of the WRO cell line as a thyroid cancer cell line is not completely certified.

For the K1 cell line, a mutation in the *PI3KCA* gene has been reported previously. By sequencing, the presence of a G1624A substitution in codon 542 (E542K) was confirmed in accordance with previously published data (Garcia-Rostan et al., 2005;

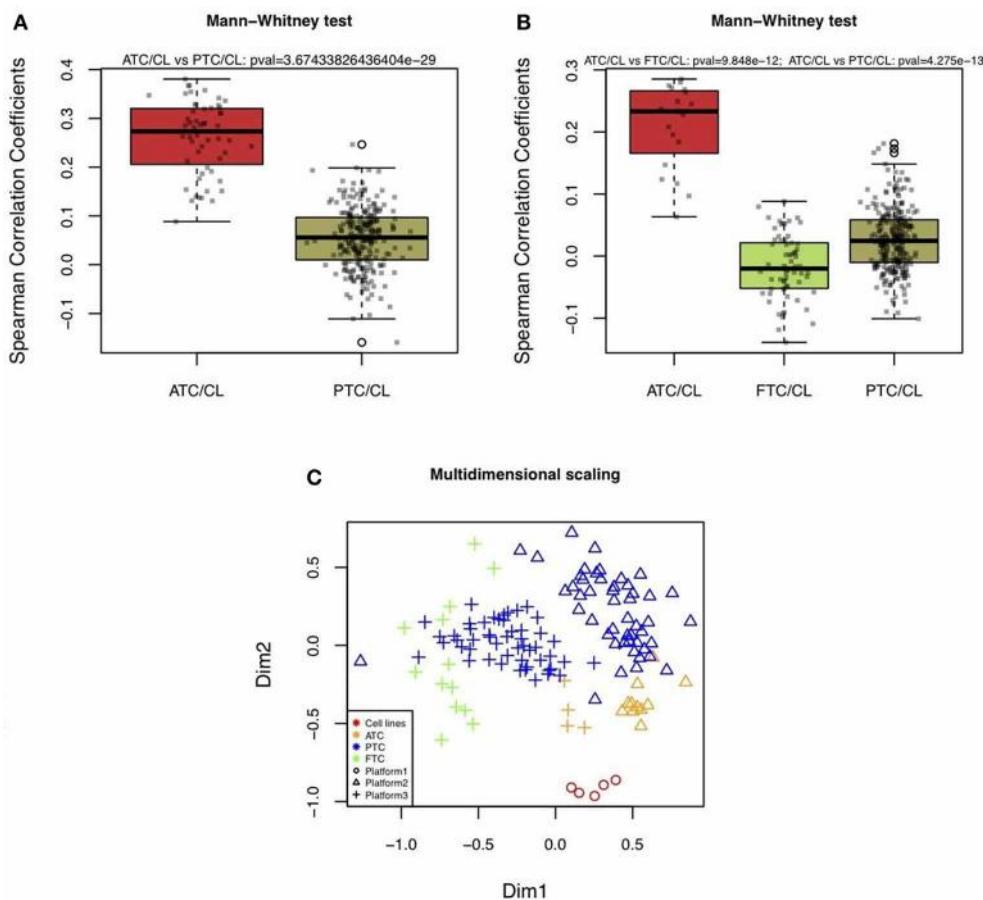


FIGURE 2 | Boxplots of correlations between cell lines and *in vivo* thyroid tumors. (A and B) Correlations were calculated based on 2,392 mRNA expressions comparing human thyroid cancer cell lines (CL) with different types of *in vivo* human thyroid tumors by using three datasets. The first was composed of five thyroid cancer cell lines (WRO, FTC133, BCPAP, TPC1, 8505C) hybridized on IRIBHM custom slides. The second was composed of 11 ATC and 49 PTC hybridized on Affymetrix HG U133A (B). *P*-values indicate whether ATC and cell lines had more similar profiles than FTC or PTC and cell lines. *P*-values were significant for both independent datasets.

Plus 2.0 (A). The third was composed of 4 ATC, 13 FTC, and 51 PTC hybridized on Affymetrix HG U133A (B). *P*-values indicate whether ATC and cell lines had more similar profiles than FTC or PTC and cell lines. *P*-values were significant for both independent datasets.

(C) 2D-multidimensional scaling representation of the mRNA expression profiles of cell lines, ATC, PTC, and FTC based on 2392 mRNA expressions.

Meireles et al., 2007). In another publication, Ricarte-Filho et al. (Ricarte-Filho et al., 2009) reported the presence of a E545K (G1633A) mutation in these cells. We could observe a glutamic acid at position 545. Thus, the G1633A mutation might potentially exist but was never described as a new mutation by Ricarte-Filho et al.

PATHWAY ANALYSIS OF THE CELL LINES

Pathway analysis of mRNA expression profiles in WRO, FTC133, BCPAP, TPC1, and 8505C cell lines was performed. In general, the cell lines abrogate gene expressions which are not necessary for their propagation in *in vitro* cultures and express genes that promote in growth and survival. The common loss of MHC2 biosynthesis and, in some cell lines, of the immune response, can be explained because they would be not necessary for a cell in culture. The common downregulation of the negative regulation of the cell death/apoptosis pathway might be explained by an ineffective feedback resulting from an excess of proliferation.

Except for FTC133 cells, a common overexpression of ribosomal biosynthesis and translation pathways were observed. This may reflect the increase in proliferation of the cancer cells induced by gene mutations. However, some recent studies suggest that the up-regulation of the ribosomal biosynthesis may induce cancer by a downregulation of the cell tumor suppressor potential (Montanaro et al., 2012). For our data, the two explanations are possible. However, the growth rate of the FTC133 cell line is higher than for the other cell lines [Dom et al. (submitted)]. For this cell line, an overexpression of the global RNA processing including alternative splicing was observed. This might allow the strong increase in proliferation of this cell line.

CORRELATIONS BETWEEN mRNA EXPRESSION PROFILES OF *in vitro* THYROID CANCER CELL LINES AND mRNA EXPRESSION PROFILES OF *in vivo* THYROID TUMORS

The correlations of the mRNA expression profiles between cell lines and ATC were significantly higher than those compared

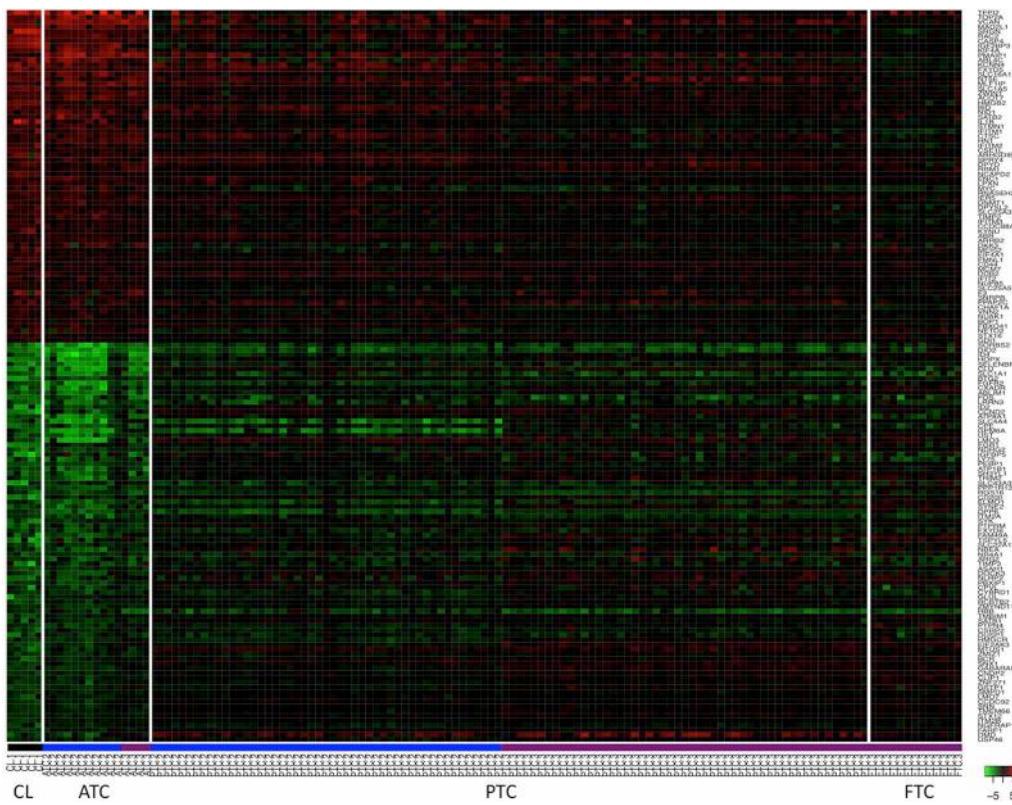


FIGURE 3 | Heatmap of 145 mRNA expressions in thyroid cancer cell lines (CL) and in human thyroid tumors. These genes were the most commonly up and down-regulated genes in ATC and in the cell lines. The level of modulation of these genes in PTC and FTC are also shown. Profiles

of the five cell lines are underlined in black. Profiles of the samples hybridized on Affymetrix HG U133 Plus 2.0 platform and on Affymetrix HG U133A platform are underlined in blue and violet respectively. Genes are indicated by their official gene symbols.

to other *in vivo* differentiated thyroid tumors (PTC, FTC). By using data derived from different microarray platforms and from two different laboratories, thereby including a larger number of *in vivo* tumors, the current data confirm our previous finding that mRNA expression profiles from human thyroid tumor cell lines with different origins are the closest to undifferentiated *in vivo* tumors (van Staveren et al., 2007). This is explained by an *in vitro* evolution of the cell lines that should be taken into account when extrapolating results obtained from these cells. An *in vitro* cell line is the result of the selection of the most rapidly proliferating and resistant cells during numerous passages. Cell lines contain one cell type, whereas *in vivo* tumors are complex mixtures of various cell types. Therefore, it might be argued that the comparisons between thyroid cancer cell lines and *in vivo* thyroid tumors could be affected by the presence of different cell types such as for instance lymphocytes, endothelial cells. However, in this study only genes were compared that were expressed in thyrocytes, i.e., were expressed in both thyroid cancer cell lines and in primary thyrocytes. This should reduce the impact of the potential presence of different cell types but, unfortunately, it cannot assure the complete elimination of a background due to commonly expressed genes in the different cell types.

ANALYSIS OF THE GENES COMMONLY AND OPPositELY MODULATED IN ATC *in vivo* AND CANCER CELL LINES

Genes commonly up-regulated in ATC and cell lines were involved in DNA replication and DNA metabolic processes. The correlation, based on mRNA expression profiles, between cell lines and ATC thus seems to be due to the strong proliferation capacity of these two types of cells. Indeed, ATC is one of the most proliferating human tumors and human thyroid cancer cell lines have a strong proliferation capacity. In our data this property was linked to ribosomal biosynthesis and translation pathways for four cell lines. As expected, the genes found regulated in an opposite direction between ATC and cell lines might reflect the differences between *in vivo* tumors and *in vitro* cultures. They are involved in the MHC class II pathway or in cell adhesion. As mentioned above in the pathway analysis of the cell lines, they abrogate gene expressions which are not necessary for their survival and growth in *in vitro* cultures. The increase of those genes in ATC, and the implication of this increase to growth and survival of the tumor *in vivo*, would be interesting to explore. However, there are a number of factors which could also explain this increase. For instance, the potential presence of immune cells, which express MHC class II in the tumor or the activation of the MHC class II pathway in these cells by an inflammatory process,

might explain an increase in these genes in ATC. In line with this, thyrocytes indeed can express MHC class II after an inflammatory stimulation and inflammation is often associated with tumor development. Furthermore, the increase of expression might also be due to a combination of the factors.

CONCLUSIONS

In this work, the aim was to further investigate the characteristics of human thyroid cancer cell lines derived from different origins. First, genetic alterations in six cell lines were in line with literature data and furthermore, the BRAF mutation in WRO cells questions this cell line as a model for FTC, indeed this mutation does not occur in FTC. Second, in five cell lines the MHC class II pathway was down-regulated and in four of them, ribosome biosynthesis and translation pathways were up-regulated. Third, by using microarray data from ATC, PTC, and FTC, we have preliminary found that mRNA expression profiles of human thyroid cancer cell lines, originally derived from ATC, poorly differentiated PTC, FTC, or PTC, were closer to ATC. Specifically, we found genes commonly up-regulated in ATC involved in DNA replication which were also up-regulated in the thyroid cancer cell lines, in accordance with their higher proliferation rate *in vitro*. In summary, these results would suggest that some human PTC or FTC derived cell lines (i.e., TPC-1) might have partially lost their original DNA synthesis/replication regulation mechanisms during their *in vitro* cell adaptation/evolution. Also, further comparisons using microarray data set from poorly differentiated thyroid cancers are needed and could be a near future goal in our laboratory.

During our study, the origin of the WRO cell line as thyroid cancer cell line has been questioned. However, the pathway analysis and the comparisons of expression profiles showed similar

results for WRO cell line and the other thyroid cancer cell lines. To address the origin of the WRO cell line, more complete analyses (including cell lines from different cancer types) could be a near future goal in our laboratory.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Cancer_Endocrinology/10.3389/fendo.2012.00133/abstract

Supplementary information A | DAVID pathway analysis of the genes modulated in each human thyroid cancer cell line.

Up: analysis of pathways using all the upregulated genes ($>0.58 \log_2$) in the cell line. Down: analysis of pathways using all the downregulated genes ($<-0.58 \log_2$) in the cell line.

Supplementary information B | DAVID pathway analysis of the genes commonly and oppositely modulated in human thyroid cancer cell lines and ATC.

Up: analysis of pathways using the 61 up-regulated genes that were upregulated in both. Down: analysis of pathways using the 84 genes down-regulated in both ATC and cell lines. Modulation: analysis of pathways using the 18 genes that were regulated in an opposite direction comparing cell lines with ATC.

Supplementary information C | DNA profiling by STR analysis of the K1 cell line.

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