



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 glycosylphosphatidylinositol-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 contralateral 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/

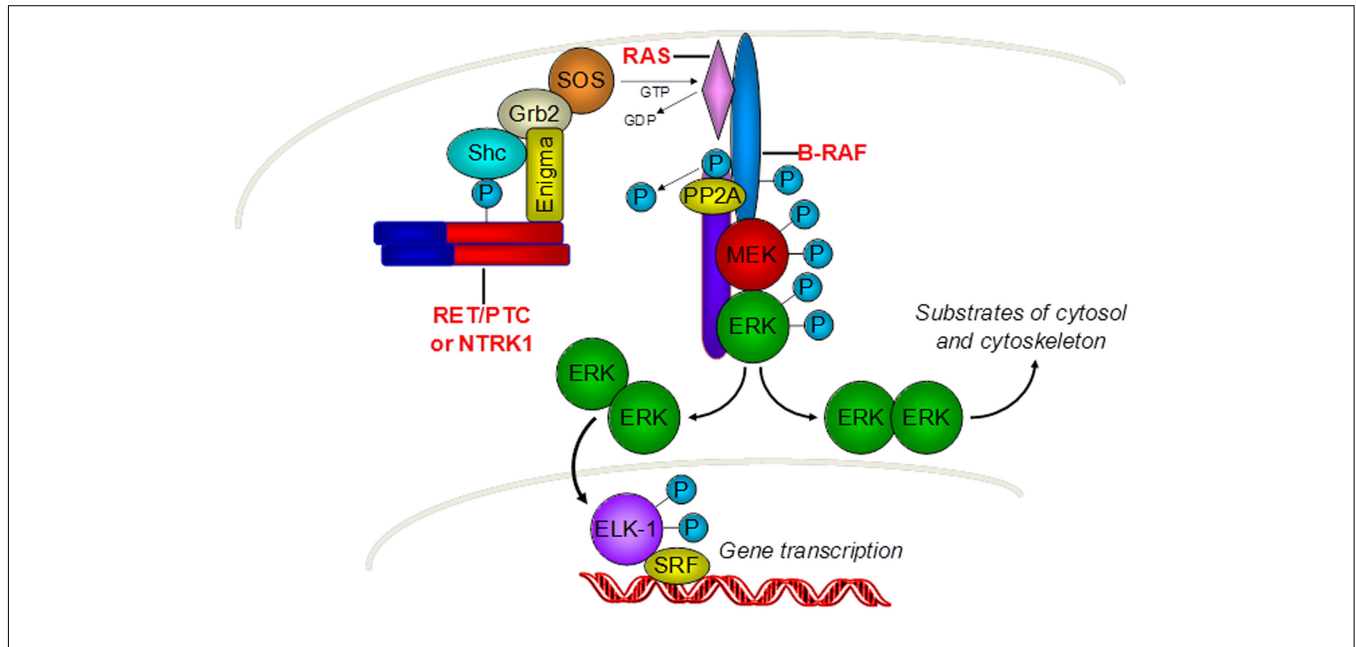


FIGURE 1 | Gene mutations involved in PTC carcinogenesis. PTC is driven by genetic alterations leading to the activation of the mitogen-activated protein kinase (MAPK) signaling pathway. Those include *RET/PTC* and *NTRK1* rearrangements and *RAS* and *BRAF* point mutations.

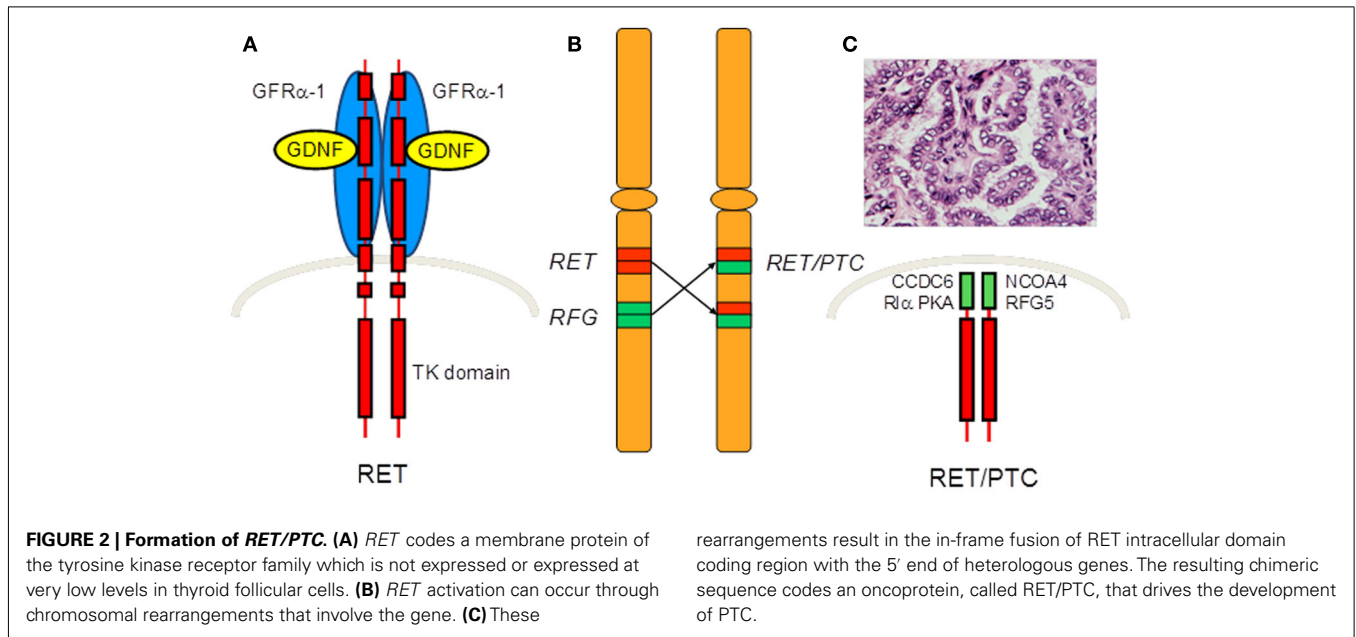


FIGURE 2 | Formation of RET/PTC. (A) *RET* codes a membrane protein of the tyrosine kinase receptor family which is not expressed or expressed at very low levels in thyroid follicular cells. (B) *RET* activation can occur through chromosomal rearrangements that involve the gene. (C) These rearrangements 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 *RF5/ELE1/ARA70*; Grieco et al., 1990; Santoro et al., 1994). *RET/PTC1* and *RET/PTC3* are intrachromosomal 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

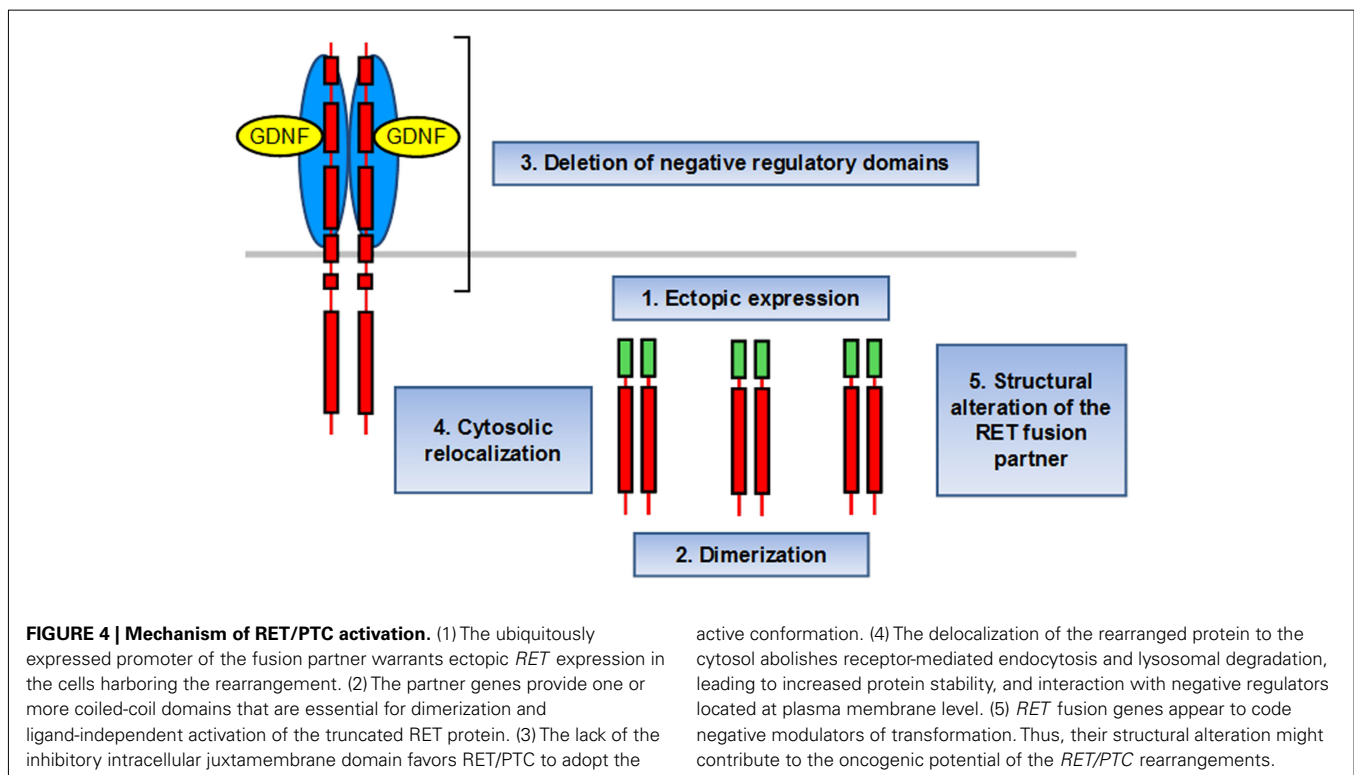
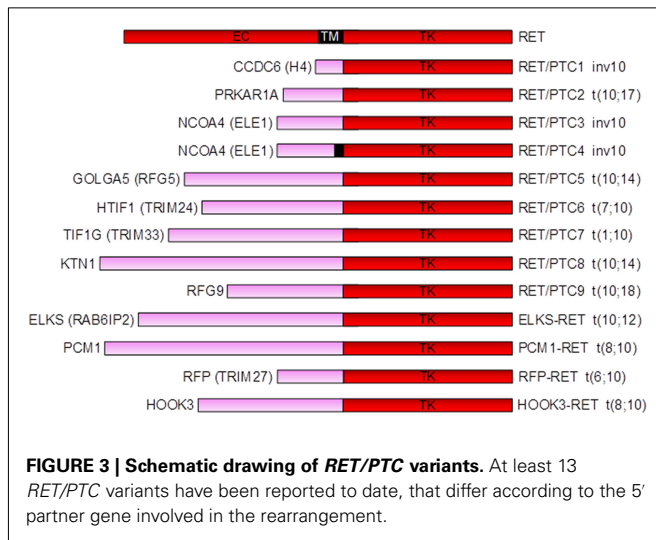
rare types of *RET/PTC* are inter-chromosomal translocations (Castellone and Santoro, 2008).

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 Phosphatase 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



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 (Bongarzzone 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); *PMCI* (participating in *PMCI-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

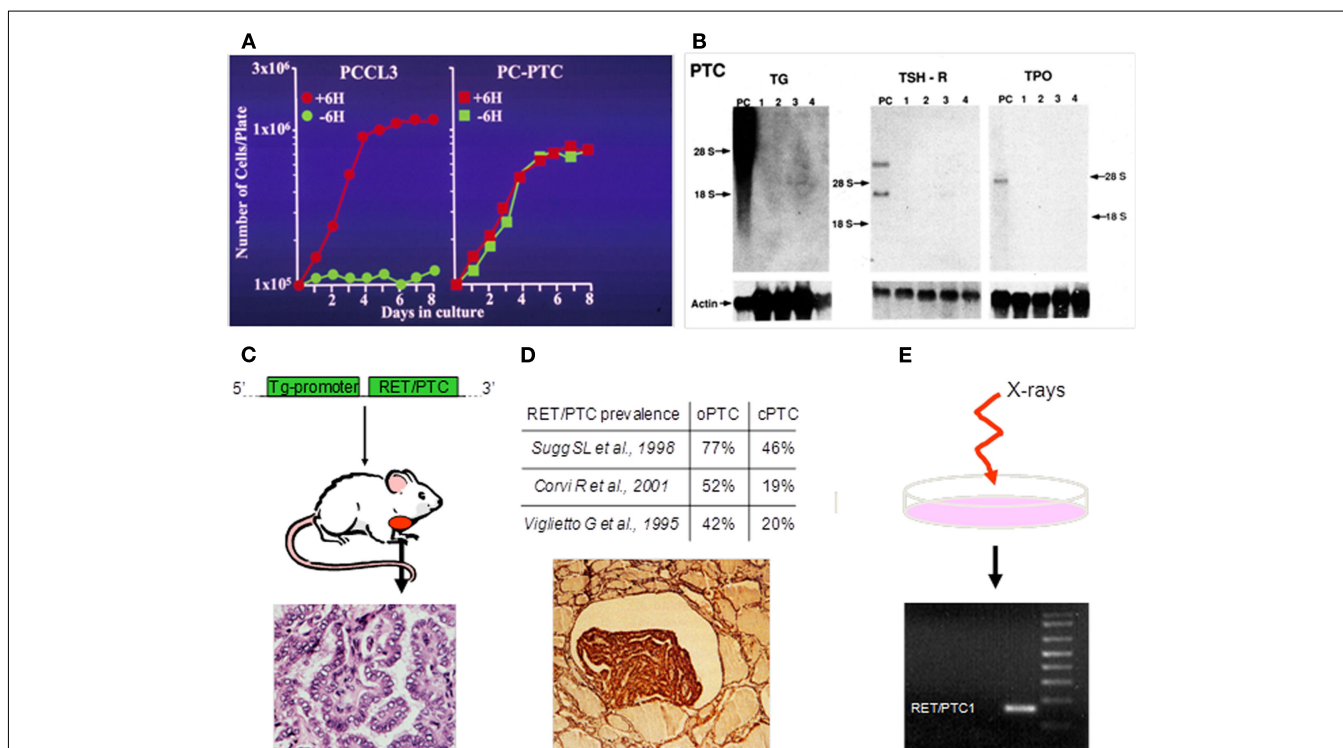


FIGURE 5 | Lines of evidence pointing to *RET/PTC* as a key first step in thyroid cancer pathogenesis. (A) *RET/PTC* expression causes TSH-independent proliferation in thyroid follicular cells. PCCL3: Parental well differentiated rat thyroid cell line. PC-PTC: PCCL3 cells stably expressing *RET/PTC1*. +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 ¹³¹I, 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/>

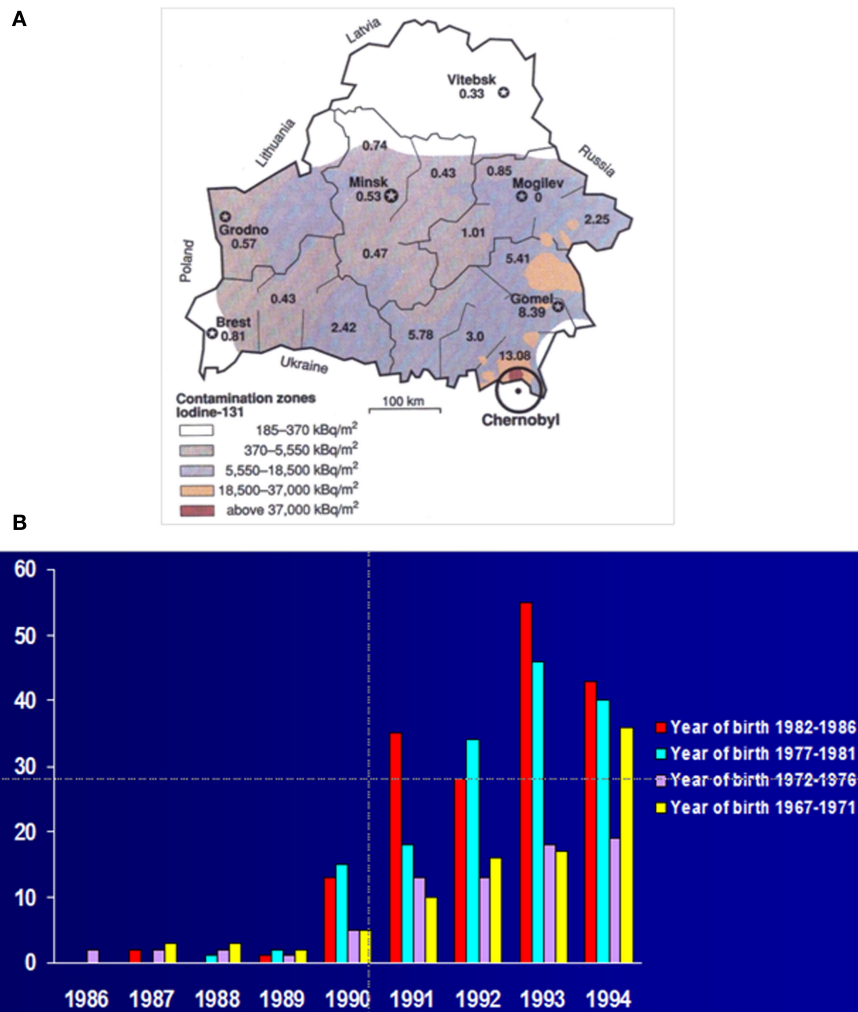


FIGURE 6 | Effects of the Chernobyl accident. (A) The Chernobyl nuclear power plant accident on April 26th 1986 resulted in the release of large amounts of iodine isotopes, mainly ¹³¹I, that contaminated the ground of surrounding regions, specially Belarus, with widespread exposure of the thyroids of 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 paid 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 cancerogenic 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 multiclonality 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 Hürthle 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 (Jiang 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-azacytidin, 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, microhomology 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

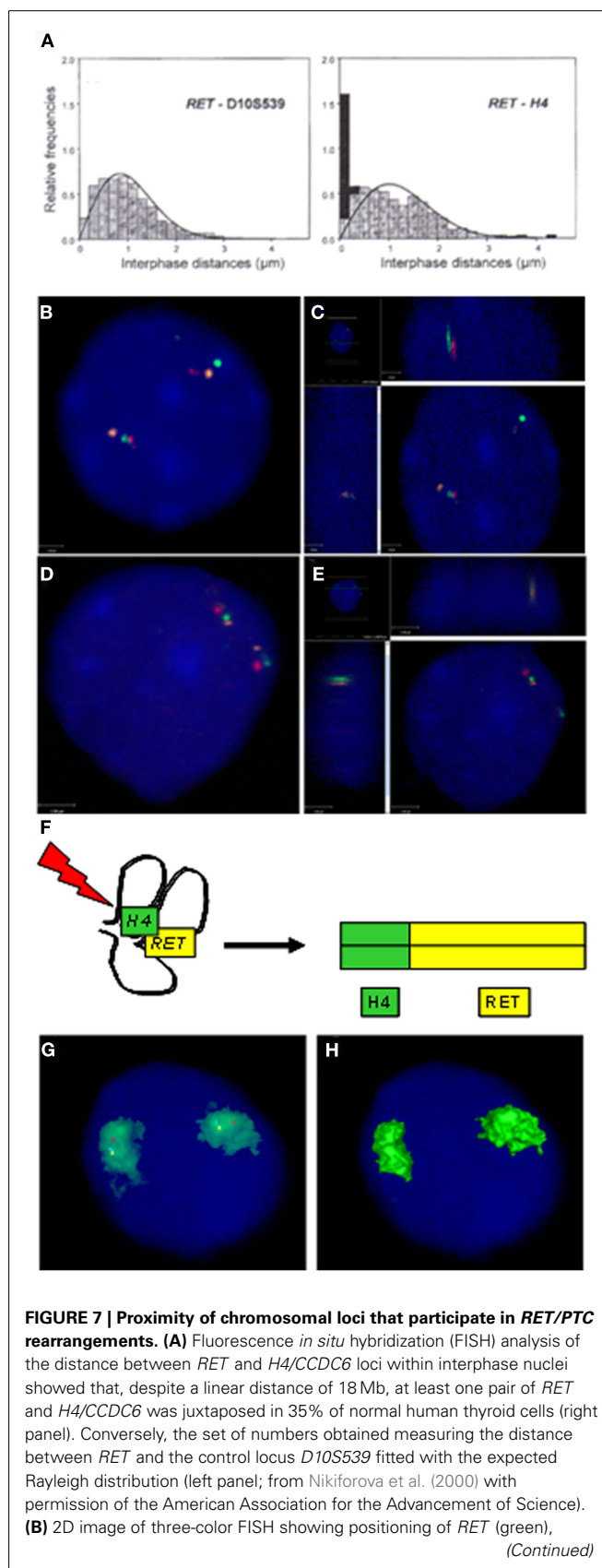


FIGURE 7 | Continued

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* TK *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

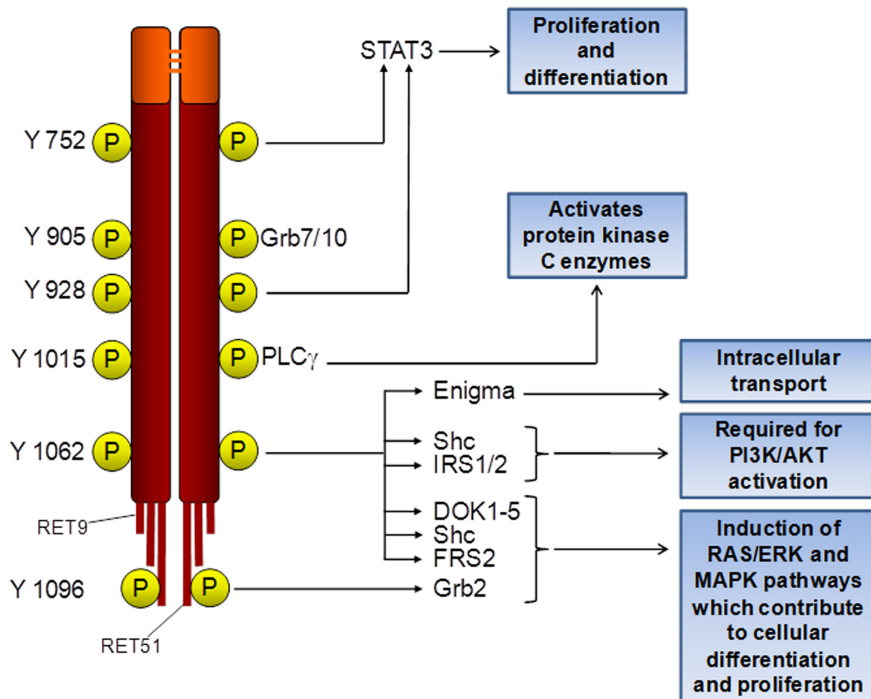


FIGURE 8 | Signal transduction pathways regulated by RET/PTCs. Tyrosine residues (Y) are numbered according to integral RET amino acid sequence. RET9 and RET51 represent splicing variants of RET C-terminal tail.

(*Cdh6*, *Fn1*), cell cycle (*Il24*), immune and inflammatory response (*Cxcl10*, *Scya2*, *Il6*, *Gbp2*, *Oas1*, *Tap1*, *RT1Aw2*, *C2ta*, *Irf1*, *Lmp2*, *Psmc2*, *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

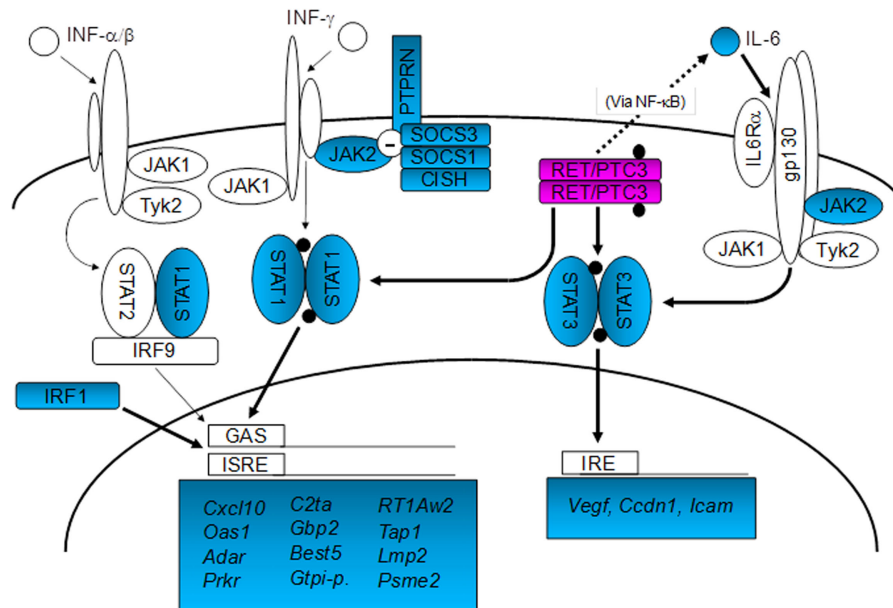


FIGURE 9 | Schematic map of the relationships between RET/PTC3 and IFN- and cytokine-activated signal transduction pathways according to the microarray results and to the literature. Up-regulated genes are shaded, whereas black dots indicate activation by phosphorylation. RET/PTC3 induces the expression 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 IL-6 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 oncoproteins 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 chemo-attractants *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.,

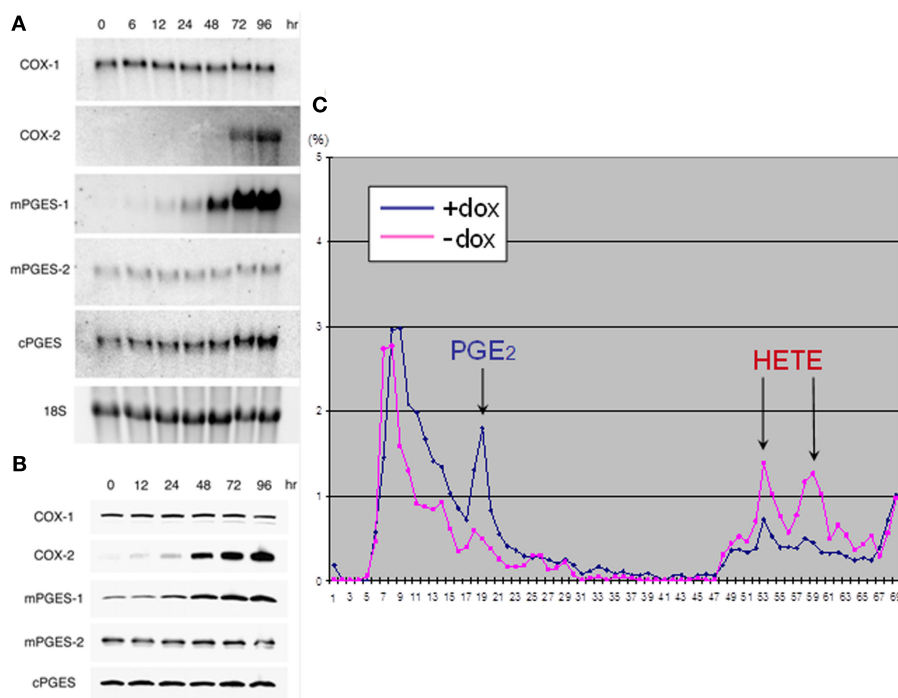


FIGURE 10 | RET/PTC3 regulates PGE₂ biosynthesis in PCCL3 cells characterized by the doxycycline (DOX)-inducible expression of the oncogene (PTC3-5 cells). (A) Northern blots showing that treatment with DOX for up to 96 h induces COX-2 and mPGES-1 mRNA but not COX-1, mPGES-2, or cPGES. **(B)** Western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