Thyroid Cancer: Role of RET and Beyond

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Abstract
Specific thyroid cancer histotypes, such as papillary and medullary thyroid carcinoma, display genetic rearrangements or point mutations of the RET gene, resulting in its oncogenic conversion. The molecular mechanisms mediating RET rearrangement with other genes and the role of partner genes in tumorigenesis have been described. In addition, the RET protein has become a molecular target for medullary thyroid carcinoma treatment.

Key Words
RET • Receptor tyrosine kinase • Thyroid cancer • Chromosome rearrangements • Tyrosine kinase inhibitors

Introduction
The RET (rearranged during transfection) gene is located on chromosome 10q11.2 straddling a region of around 55,000 bp and is composed of 21 exons. The gene codes for a transmembrane protein belonging to the receptor tyrosine kinases family [1].

The RET protein is composed of three different domains: the extracellular domain, transmembrane domain and intracellular domain, and varies in length due to a 3' end alternative splicing that generates three different isoforms containing 9 (RET9), 43 (RET43) or 51 (RET51) amino acids behind the RET G1063 residue [1, 2]. RET9 and RET51 are the most represented isoforms in vivo. The extracellular segment of RET protein is N-glycosylated and contains four Ca$^{2+}$ dependent cell adhesion (cadherin)-like domains followed by a cysteine-rich domain [3, 4]. N-glycosylation of the extracellular domain occurs in the endoplasmic reticulum and Golgi apparatus and is necessary for transport to the plasma membrane. Indeed, only the fully mature glycosylated 170-kDa isoform of RET is exposed on the cell surface while the highly mannose-rich 150-kDa isoform is confined to the Golgi apparatus [5]. The transmembrane segment is composed of 22 amino acids. The intracellular portion contains the tyrosine kinase domain, divided into two subdomains by the insertion of 27 amino acids, and a COOH terminal tail.

RET functions as the receptor of the four members of the glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) which include GDNF itself, neurturin, artemin and persephin [4]. GFLs are presented to RET by GPI (glycosylphosphatidylinositol)-anchored coreceptor molecules, named GFRα1–4, each binding preferentially one of the four different GFLs.
RET binding to any GFL-GFRα complex induces receptor dimerization, which in turn triggers transphosphorylation of specific tyrosine (Y) residues located in the tyrosine kinase domain and in the COOH-terminal tail of each RET monomer. Phosphorylation of such tyrosines initiates an intracellular signaling cascade that culminates in the activation of several cellular programs such as motility, survival and proliferation [6].

Several tyrosines have been identified as RET auto-phosphorylation sites [7, 8]. In particular, Y900 and Y905 are located in the activation loop of the kinase and their phosphorylation is necessary for stabilization of RET in an active conformation, while RET Y1062 is located in the COOH-tail and is a multidocking site for many signaling molecules containing a phosphotyrosine-binding domain such as SHC, N-SHC (RAI), FRS2 and IRS1/2 [9–15]. Binding of these proteins contributes to activate the two main RET signaling pathways, i.e. the RAS/MAPK and the PI3K/AKT ones.

RET is expressed mostly in tissues of neuroectodermal origin, such as the enteric ganglia, the adrenal chromaffin cells and thyroid C cells; in sensory and autonomic ganglia of the peripheral nervous system; in a subset of central nervous system nuclei; in the kidney during embryonic and fetal stage; and in testes germ cells [6, 16]. RET and its ligands play a significant role in the development of all these structures as shown by the RET-null mice phenotype featuring absence of the superior cervical ganglia and enteric nervous system, kidney agenesis and reduction of thyroid C cells, as well as impaired spermatogenesis due to lack of spermatogonial stem cell renewal and differentiation [6, 16]. In accordance, individuals with germline loss-of-function mutations of RET are affected by congenital megacolon or Hirschsprung disease characterized by intestinal aganglionosis [17].

Gain-of-function mutations of the RET gene have been identified in two different types of thyroid neoplasia: medullary thyroid carcinoma (MTC) and papillary thyroid carcinoma.

**RET Mutations in MTC: A New Target for Cancer Treatment**

MTC arises from neural crest-derived calcitonin-producing thyroid parafollicular C cells and represents 5–10% of all thyroid cancers. Although most MTCs are sporadic and affect adult patients, around 25% of cases are familial and occur in the frame of inherited cancer syndromes called multiple endocrine neoplasia type 2 (MEN2) syndromes (OMIM: No. 171400) and may have an early onset [18].

MEN2 syndromes comprise three different kinds: MEN2A, MEN2B and familial MTC (FMTC). MEN2A and B phenotypes are depicted by a plethora of tumors that, with different combinations, arise from tissues of neuroectodermal origins. All patients display MTC, associated in 50% of cases to pheochromocytoma arising from chromaffin cells of adrenal medulla. Additional features are parathyroid hyperplasia and hereditary localized pruritus in MEN2A, ganglioneuromatosis of the intestine, thickening of corneal nerves and marfanoid habitus in MEN2B. FMTC consists in the isolated occurrence of MTC and is nowadays regarded as a phenotypic variant of MEN2A with decreased penetrance and/or delay of the other neoplastic manifestation [19, 20].

In over 90% of cases, MEN2 syndromes are due to germline missense mutations of the RET gene. There is partial overlap between the type of mutation and the kind of phenotype displayed by patients. Thus, the majority of MEN2A and FMTC carriers bear mutations of one of the cysteines in the extracellular cysteine-rich domain of RET (most frequently, C634). FMTC is also associated with changes in the N-terminal (E768D, L790F, Y791F, V804L, V804M) or C-terminal (S891A) lobes of RET kinase. The vast majority of MEN2B patients display the M918T mutation in RET kinase domain, whereas only a small fraction harbors the A883F substitution. In addition, somatic mutations of RET E768, V804 and mainly M918 are found in approximately 40% of sporadic MTC cases and correlate with a bad prognosis [20–23]. Over the years many new germline RET mutations have been identified due to the systematic screening of MTC patients; however, the pathological meaning of such variants is not always clear due to lack of information about mutation cosegregation with disease within families and of functional studies. A comprehensive database annotating all RET variants has been generated [24].

The mechanism through which MEN2-associated mutations switch on RET oncogenic activity is strictly dependent on the location of the amino acid change. Extracellular cysteine mutants display constitutive kinase activity consequent to disulfide bond-mediated homodimerization (fig. 1). Mutations associated with FMTC, which in cases target cysteine residues other than C634, are less potently transforming than MEN2A-associated mutations, possibly because of their lower activity to induce RET S-S linked dimers [25, 26]. Constitutive activation as well as a change in substrate specificity has
been implicated in the MEN2B-associated substitution [27]. In line with this model, MEN2B mutants differ from MEN2A mutants in the stoichiometry of phosphorylation of RET tyrosines and of various intracellular proteins [28, 29]. Moreover, MEN2B-expressing tumors have different gene expression profiles as compared to MEN2A-expressing tumors [30]. The mechanisms through which RET intracellular mutations (other than M918T) constitutively activate RET enzymatic function has not been clearly elucidated.

In 2009, the American Thyroid Association created specific MTC clinical guidelines consisting of 122 evidence-based recommendations to assist in the clinical care of MTC patients [31]. The guidelines included germline genetic testing in all MTC patients to distinguish sporadic from familial cases and to classify patients in different disease risk levels ranging from A (most severe) to D (less severe) on the basis of RET mutation. The risk level classification is relevant for making decisions with respect to prophylactic thyroidectomy and intraoperative amputation of the parathyroid glands.

Although identification of RET mutations in MEN2 carriers has drastically improved clinical management of patients for preventive or early surgery, most MTC cases come to diagnosis when the disease is already metastatic and little can be done because of the resistance that MTC displays towards conventional chemotherapy and radiotherapy [32]. Therefore, identification of novel treatments for MTC patients appears to be mandatory. The past 10 years have witnessed the advent of new classes of antineoplastic drugs whose mechanism of action is based on targeting of tyrosine kinases causally involved in cancer [33]. These drugs include monoclonal antibodies such as Herceptin (trastuzumab) or Erbitux (cetuximab) directed against HER2 and HER1 receptor tyrosine kinases and used in breast and colon cancer treatment, respectively.

Another important class of targeted therapies is represented by small molecule tyrosine kinase inhibitors, which compete with ATP, thereby obstructing autophosphorylation and signal transduction downstream from the targeted kinase. Prominent examples are STI571 [Gleevec (imatinib)] against BCR-ABL in chronic myeloid leukemia and against c-KIT and PDGFR in gastrointestinal stromal tumors [33], and ZD1839 [Iressa (gefitinib)] against epithelial growth factor receptor (EGFR) in non-small cell lung carcinoma [34]. RET is a critical target for medullary thyroid cancer treatment [32]. Several small molecules are known to exert RET inhibition. Virtually all of them are believed to block the kinase by competing with ATP.

Two pyrazolopyrimidines (PP1 and PP2) showed half-maximal RET inhibitory concentrations in the nM range (≤100 nM) and were able to block RET’s oncogenic effects in cell cultures [35, 36]. PP1 also induced RET protein destruction through proteosomal degradation [37]. The 2-indolinone RPI-1 was effective against RET, but only at high doses (IC_{50} = 3.6 μM for cell proliferation) and ex-
erted in vivo antitumor effects [38]. Two indolocarbazole derivatives, CEP-701 and CEP-751, inhibited RET-MEN 2A oncoproteins at nM concentrations. Importantly, these compounds also inhibited tumor growth in MTC cell xenografts [39]. Nevertheless, none of these compounds has been evaluated in clinical studies due to their toxicity or limited bioavailability.

Another group of anti-RET compounds that had been tested in phase I clinical trials has been described. Such molecules comprise vandetanib, sorafenib, sunitinib, cabozantinib and lenvatinib [40–43]. All these compounds have been evaluated in clinical trials for efficacy in MTC treatment. The anilinoquinazoline vandetanib, also known as ZD6474, inhibits RET with an IC50 of 100 nM and has been the first anti-RET agent to be FDA approved for MTC treatment on the basis of the results exerted in clinical trials for MTC treatment [44]. ZD6474 is also a potent inhibitor of KDR, the vascular endothelial growth factor receptor and EGFR. Inhibition of KDR potentiates the antineoplastic activity through an antiangiogenic effect, whereas EGFR inhibition might be important to impede the complementation of RET inhibition via EGFR hyperactivation [40, 45]. Mutations in codons 804 and 806 in the ATP-binding pocket have been shown to confer resistance to vandetanib, which may be a concern for secondary resistance to the drug [46, 47]. In a phase III clinical trial, vandetanib was able to increase progression-free survival, inducing a partial response in around 20% of patients and a stabilization of disease in 50% of treated cases compared to control [44].

### RET in Papillary Thyroid Carcinoma: A New Role for Partner Genes

Papillary thyroid carcinoma (PTC) is the most frequent thyroid cancer and consists in a well-differentiated carcinoma, originating from thyroid follicular cells and associated to exposure to ionizing radiation [48, 49]. Consistently, typical molecular features of PTCs are chromosomal aberrations generated as a consequence of ionizing radiation-induced double-strand breaks and unfaithful repair. In particular, PTCs display chromosomal rearrangements of chr. 10q, causing the rupture of the RET gene and its fusion to heterologous genes due to unfaithful repair [50]. The partner genes encode heterogeneous proteins all containing protein-protein interaction domains such as coiled-coil motifs [51]. RET/PTC1 and RET/PTC3 represent over 90% of all RET/PTC rearrangements identified so far. In both cases, the chromosomal aberration consists in a paracentric inversion of the long arm of chromosome 10 where, together with RET the corresponding fusion partner of RET/PTC1, the vascular endothelial growth factor receptor and EGFR. Inhibition of KDR potentiates the antineoplastic activity through an antiangiogenic effect, whereas EGFR inhibition might be important to impede the complementation of RET inhibition via EGFR hyperactivation [40, 45]. Mutations in codons 804 and 806 in the ATP-binding pocket have been shown to confer resistance to vandetanib, which may be a concern for secondary resistance to the drug [46, 47]. In a phase III clinical trial, vandetanib was able to increase progression-free survival, inducing a partial response in around 20% of patients and a stabilization of disease in 50% of treated cases compared to control [44].

### Table 1. RET/PTC rearrangements

<table>
<thead>
<tr>
<th>Fusion name</th>
<th>RET partner</th>
<th>Function</th>
<th>Chromosome rearrangement</th>
</tr>
</thead>
<tbody>
<tr>
<td>RET/PTC1</td>
<td>H4/D10S170</td>
<td>apoptotic factor</td>
<td>inv10(q11.2;q21)</td>
</tr>
<tr>
<td>RET/PTC2</td>
<td>PKA RIIa</td>
<td>regulatory subunit of protein kinase A</td>
<td>t(10;17)(q11.2;q23)</td>
</tr>
<tr>
<td>RET/PTC3</td>
<td>RFG/ELE1/NcoA4</td>
<td>coactivator of nuclear receptor</td>
<td>inv10(q11.2;q10)</td>
</tr>
<tr>
<td>RET/PTC4</td>
<td>RFG/ELE1/NcoA4</td>
<td>coactivator of nuclear receptor</td>
<td>inv10(q11.2;q10)</td>
</tr>
<tr>
<td>RET/PTC5</td>
<td>RFG5/Golgin 84</td>
<td>Golgi structure maintenance</td>
<td>t(14;10)(q11.2;q32)</td>
</tr>
<tr>
<td>RET/PTC6</td>
<td>HTIF1</td>
<td>coactivator of nuclear receptor</td>
<td>t(7;10)(q32–34;q11.2)</td>
</tr>
<tr>
<td>RET/PTC7</td>
<td>RFG7/HTIF3/TRIMM33</td>
<td>coactivator of androgen receptor and SMAD4-E3 ligase</td>
<td>t(1;10)(p13;q11.2)</td>
</tr>
<tr>
<td>RET/PTC8</td>
<td>KNT1/kinecin</td>
<td>regulator of intracellular motility</td>
<td>t(1;10)(q11.2;q22.1)</td>
</tr>
<tr>
<td>RET/PTC9</td>
<td>RFG9</td>
<td>unknown</td>
<td>t(10;18)(q11.2;q21–22)</td>
</tr>
<tr>
<td>RET/ELK5</td>
<td>ELK5</td>
<td>NF-κB regulatory protein</td>
<td>t(10;12)(q11.2;p13.3)</td>
</tr>
<tr>
<td>RET/PCMI</td>
<td>PCMI</td>
<td>centrosome assembly protein</td>
<td>t(8;10)(p21–22;q11.2)</td>
</tr>
<tr>
<td>RET/RFP</td>
<td>RFP/TRIMM27</td>
<td>PML interactor and RB modulator</td>
<td>t(6;10)(p21;q11.2)</td>
</tr>
<tr>
<td>RET/HOOK3</td>
<td>HOOK3</td>
<td>microtubule-binding protein</td>
<td>t(8;10)(p11.21;q11.2)</td>
</tr>
</tbody>
</table>

Papillary thyroid carcinoma (PTC) is the most frequent thyroid cancer and consists in a well-differentiated carcinoma, originating from thyroid follicular cells and associated to exposure to ionizing radiation [48, 49]. Consistently, typical molecular features of PTCs are chromosomal aberrations generated as a consequence of ionizing radiation-induced double-strand breaks and unfaithful repair. In particular, PTCs display chromosomal rearrangements of chr. 10q, causing the rupture of the RET gene and its fusion to heterologous genes due to unfaithful repair [50]. The partner genes encode heterogeneous proteins all containing protein-protein interaction domains such as coiled-coil motifs [51]. RET/PTC1 and RET/PTC3 represent over 90% of all RET/PTC rearrangements identified so far. In both cases, the chromosomal aberration consists in a paracentric inversion of the long arm of chromosome 10 where, together with RET the corresponding fusion partner of RET/PTC1, the vascular endothelial growth factor receptor and EGFR. Inhibition of KDR potentiates the antineoplastic activity through an antiangiogenic effect, whereas EGFR inhibition might be important to impede the complementation of RET inhibition via EGFR hyperactivation [40, 45]. Mutations in codons 804 and 806 in the ATP-binding pocket have been shown to confer resistance to vandetanib, which may be a concern for secondary resistance to the drug [46, 47]. In a phase III clinical trial, vandetanib was able to increase progression-free survival, inducing a partial response in around 20% of patients and a stabilization of disease in 50% of treated cases compared to control [44].
ubiquitously active and drive RET expression in thyroid cells in vitro. The best described mechanism for RET/PTC oncogenic conversion resides in the ligand-independent activation of RET kinase as a consequence of its fusion to heterologous proteins, such as CCDC6 and NCOA4. The independence from ligand binding is achieved through removal of negatively regulating sequence from wild-type RET proteins such as the extracellular and juxtamembrane domains as well as its joining to protein homodimerization motives contained in the fusion partners, namely coiled-coil domains. Constitutive dimerization and transphosphorylation of RET mediates the continuous activation of downstream signaling pathways. Moreover, as a consequence of gene fusion, the RET tyrosine kinase domain comes under the control of the new gene over, as a consequence of gene fusion, the RET tyrosine kinase domain comes under the control of the new gene promoter. Differently from RET, which is normally expressed in a restricted subset of neuronal and neuroectoderm-derived cells, such partner gene promoters are ubiquitously active and drive RET expression in thyroid follicular cells [16, 57] (fig. 1).

RET oncogenic signaling sustains the acquisition of several hallmarks of cancer cells including cell autonomy and independence from growth factors (like thyroid stimulating hormone), resistance to proapoptotic stimuli and motility [53, 55]. Such programs are principally switched on by the activation of the RAS/MAPK and PI3K/AKT signaling pathways through RET Y1062 auto-phosphorylation [55, 58]. Nevertheless, the low penetrance of the disease in transgenic animals, as well as the presence of such rearrangements in papillary microcarcinomas that do not progress to invasive cancer, suggests that additional oncogenic events should occur to generate a clinically relevant disease [59]. In line with these observations, RET/PTC1 and RET/PTC3 oncogenes are unable to induce a fully transformed phenotype in normal rat thyroid cells in vitro [53, 55]. Notably, the acute expression of RET/PTC1 and RET/PTC3 in thyroid cells in vitro has been shown to activate a proapoptotic response due to unscheduled activation of the RAS/MAPK pathway [60, 61]. It is conceivable that RET/PTC rearrangements are not sufficient to induce a symptomatically evident cancer, unless cells activate antiapoptotic or antisenescence programs, through further mutational events or epigenetic modifications.

RET/PTC rearrangements are confined to the papillary histotype of thyroid carcinoma and have never been described in other tumor types [51]. The thyroid gland is exposed to ionizing radiation more than other tissues due to its ability to concentrate iodide radioactive isotopes. On the other hand, a large set of data have shown that nuclear chromatin architecture in thyroid cells bring in close proximity the CCDC6, NCOA4 and RET genetic loci, facilitating the assembly of RET/PTC1 (CCDC6-RET) and RET/PTC3 (NCOA4-RET) fusions [62–64]. Importantly, in other cancers, also displaying chromosomal rearrangements such as prostate carcinoma, the spatial organization of chromatin has been shown to be dictated by the action of transcriptional factors that somehow guide the relevant genes in the same nuclear neighborhood, promoting their fusion [65]. Not all RET/PTC rearrangements seem to be linked to radiation exposure, especially those displaying RET/PTC1 rearrangement [48]. In these cases, the presence of the fragile sites FRA10C and FRA10G located on chromosome 10 next to CCDC6 and RET genes, respectively, might provide an alternative mechanism for the generation of RET/PTC fusion [66]. Thus, under specific insults such as ethanol, caffeine and hypoxia, fragile sites are hotspots of spontaneous chromosome breakage and translocation [67]. In addition, H2O2, a potent DNA damaging agent produced in large amounts by the thyroid gland during the process of thyroid hormone synthesis, is able to induce RET/PTC1 rearrangement as well, suggesting that oxidative stress might be an additional mechanism through which RET/PTC1 rearrangement might occur [68].

Many types of cancers, such as hepatocarcinoma and gastric and colon cancers, are promoted by inflammation. Hashimoto’s thyroiditis, the most common organ-specific autoimmune disease in humans, is often associated to papillary thyroid cancer [69]. Several reports have shown that RET/PTC signaling via the RAS/MAPK cascade in thyroid follicular cells endorses an inflammatory-like response through the production of several cytokines and chemokines that act in an autocrine as well as paracrine fashion, recruiting macrophages, lymphocytes and mast cells within the tumor mass and promoting cell survival, invasion and angiogenesis [55, 58, 70, 71]. More studies need to be done to clarify whether the oncogene-mediated proinflammatory response supports the development of a chronic inflammation or whether the latter facilitates the process of neoplastic transformation.

Several studies suggest that the genes most frequently rearranged to RET, CCDC6 and NCOA4 might display a tumor suppressor-like activity and their loss of function could be involved in tumorigenesis. CCDC6 (coiled-coil domain containing sequence 6, also known as H4/D10S170) gene product is a ubiquitously expressed 65kDa nuclear and cytosolic protein that has been shown to display proapoptotic activity and to be involved in the
ATM-mediated cellular response to DNA damage [72, 73]. More recently, a direct role of CCDC6 in the repression of CREB1, a transcriptional factor essential for thyroid cell growth and differentiation, has been described [74]. The CCDC6 gene has been found fused to the PDGFRβ gene in atypical chronic myeloid leukemia and to PTEN tumor suppressor phosphatase in PTCs, indicating its high susceptibility to gene fusion [75, 76]. The NCOA4 (nuclear receptor coactivator 4, also known as RFG/ELE1/ARA70) gene encodes a 70-kDa protein functioning as a coactivator of androgen receptor and PPARγ (peroxisome-proliferator activated receptor-γ) [77]. PPARγ-PAX8 gene fusion has been found in around 30% of follicular thyroid carcinoma [78]. Functional studies have demonstrated that the PAX8-PPARγ chimeric protein functions as a dominant negative inhibitor of the parental wild-type PPARγ protein which physiologically exerts antineoplastic signaling in thyroid follicular cells. NCOA4 is a PPARγ and might help this tumor suppressor function [78]. In addition, ectopic overexpression of NCOA4 in the prostate cancer cell line LNCAP reduces cell proliferation, and NCOA4 protein expression has been shown to be reduced in aggressive prostate and breast cancers with respect to normal tissue or well-differentiated carcinomas [79–81]. Finally, the chromosomal region containing the NCOA4 gene has been identified as a prostate cancer risk locus [82, 83]. A RET partner with a well-known tumor suppressor function is PRKARIA, encoding the regulatory subunit of protein kinase A, RIKα and fused to RET in RET/PTC2 rearrangement. Germline inactivating mutations of PRKARIA gene have been found in patients affected by a rare autosomal dominant cancer-prone syndrome, Carney Complex, characterized by lentiginosis, atrial and cutaneous myxoma, pituitary adenoma, testicular tumors, ovarian cystis, schwannoma, and thyroid adenoma and carcinoma [84, 85]. Somatic LOH at the gene locus on chr. 17q22–24 in Carney Complex patient tumor DNA confirms the tumor suppressor function of PRKARIA [84].

In conclusion, all these data strongly support the concept that RET partner genes’ inactivation in the context of RET/PTC rearrangements might be involved in thyroid carcinogenesis. In addition, since the rearrangement affects only one allele, it might be envisaged that chimeric RET/PTC oncoproteins might function as dominant negative mutants for their ability to interact and phosphorylate the wild-type partner proteins, as shown for CCDC6 and NCOA4 proteins [72, 86]. In this light, the function of RET gene fusion partners, possibly representing new thyroid cancer genes, should be studied.

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Disclosure Statement

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