Central Role of RET in Thyroid Cancer

Massimo Santoro and Francesca Carlomagno

Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università degli Studi di Napoli Federico II, 80131 Napoli, Italy

Correspondence: masantor@unina.it

RET (rearranged during transfection) is a receptor tyrosine kinase involved in the development of neural crest derived cell lineages, kidney, and male germ cells. Different human cancers, including papillary and medullary thyroid carcinomas, lung adenocarcinomas, and myeloproliferative disorders display gain-of-function mutations in *RET*. Accordingly, RET protein has become a promising molecular target for cancer treatment.

The human *RET* (rearranged during transfection) gene maps on 10q11.2 and is composed of 21 exons spanning a region of 55,000 bp. It encodes a single-pass *trans*-membrane protein, RET, that belongs to the receptor tyrosine kinase (RTK) family (Pasini et al. 1995). The RET extracellular segment contains four cadherin-like domains, followed by a domain containing cysteine residues involved in the formation of intramolecular disulfide bonds (Fig. 1A) (Anders et al. 2001; Airaksinen and Saarma 2002). RET protein is highly glycosylated and *N*-glycosylation is necessary for its transport to the cell surface. Only the fully mature glycosylated 170 kDa RET protein isoform is exposed to the extracellular compartment, whereas the mannose-rich 150 kDa isoform is confined to the Golgi (Takahashi et al. 1993; Carlomagno et al. 1996). The transmembrane segment is composed of 22 amino acids, among which S649 and S653 mediate self-association and dimerization of RET, possibly via formation of intermolecular hydrogen bonding (Kjaer et al. 2006). The intracellular portion of RET contains the

tyrosine kinase domain split into two subdomains by the insertion of 27 amino acids. The RET COOH-terminal tail varies in length as a result of alternative splicing of the 3' end (carboxy terminal with respect to glycine 1063), generating three different isoforms that contain 9 (RET9), 43 (RET43), or 51 (RET51) amino acids (Myers et al. 1995). RET9 and RET51 are the most abundant isoforms, and they activate similar signaling pathways through interaction with diverse protein complexes, and may exert a differential role in development (Fig. 1A) (de Graaff et al. 2001).

RET shows several autophosphorylation sites (Fig. 1A) (Liu et al. 1996; Kawamoto et al. 2004). RET tyrosine 1062 (Y1062) functions as a multidocking site for signaling molecules containing a phosphotyrosine-binding (PTB) domain (Asai et al. 1996). Phospho-Y1062 binding proteins include SHC, N-SHC (RAI), FRS2, IRS1/2, DOK1, and DOK4/5 that, in turn, contribute to the activation of RAS-MAPK (mitogen-activated protein kinases) and PI3K (phosphatidylinositol 3 kinase)-AKT pathways.

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Figure 1. Illustration of the mechanisms of activation of wild-type (wt) RET and RET-derived oncoproteins. (A) Wild-type RET activation is mediated by ligand (GFL)-induced dimerization; ligand binding to RET is not direct and mediated by GFR- α coreceptors (not shown); major RET autophosphorylation sites and downstream signaling pathways are indicated. RET extracellular cadherin-like domains are represented in red. The split intracellular RET tyrosine kinase domain, as well as the three alternative carboxy-terminal RET tails, are also depicted. (B) RET/PTC activation is mediated by coiled-coil-induced dimerization (*left*); activation of RET cysteine mutants associated with MEN2A or FMTC is mediated by disulfide bonds-mediated dimerization (*right*).

Y1096, specific to the RET51 splicing variant, couples to the PI3K-AKT and RAS-MAPK pathways, as well. These signaling cascades mediate RET-dependent cell survival, proliferation, and motility (Alberti et al. 1998; Murakami et al. 1999; Segouffin-Cariou and Billaud 2000; Melillo et al. 2001a,b; Schuetz et al. 2004). Y905 is located in the activation loop of the RET kinase and its phosphorylation is associated with RET kinase activation (Knowles et al. 2006). Finally, Y981 and Y1015 have been shown to be coupled to important signaling molecules such as SRC and PLC- γ , respectively (Borrello et al. 1996; Encinas et al. 2004).

RET is the receptor for a group of neurotrophic growth factors that belong to the glial cell line-derived neurotrophic factor (GDNF) family (GFLs, GDNF family ligands), namely, GDNF, Neurturin (NRT), Artemin (ART), and Persephin (PSF) (Airaksinen and Saarma 2002). GFLs mediate RET protein dimerization and activation (Fig. 1A). GFLs are presented to RET by GPI (glycosylphosphatidylinositol)-anchored coreceptors, called GFR- α (GDNF family receptor α 1-4). Differential tissue expression dictates the specificity of action displayed by

alternative GLF-GFR- α pairs during development and adult life (Baloh et al. 2000; Airaksinen and Saarma 2002).

Together with other membrane (DCC and p75NTR) or nuclear (androgen receptor, AR) receptors, RET belongs to the family of so-called “dependence” receptors (Mehlen and Bredesen 2011). In the absence of ligand, RET exerts a proapoptotic activity, that is blocked on ligand stimulation (Bordeaux et al. 2000). Such proapoptotic activity is RET kinase-independent and mediated by cleavage of RET cytosolic portion by caspase-3, which, in turn, releases a carboxy-terminal RET peptide that is able to induce cell death (Bordeaux et al. 2000). It is feasible that such activity is important for RET developmental function, because it may control migration of RET-expressing cells by limiting survival of cells that move beyond ligand availability (Bordeaux et al. 2000; Cañibano et al. 2007). Whether modulation of this function is also important for RET-associated diseases is still unknown. However, it is interesting to note that a cancer-associated RET mutant (RET-C634R, see below) does not exert cleavage-dependent proapoptotic effects, whereas RET mutants as-

sociated with defective development (Hirschsprung disease, see below) exert strong proapoptotic activity that is refractory to modulation by ligand (Bordeaux et al. 2000).

RET is expressed in enteric ganglia, adrenal medulla chromaffin cells, thyroid C cells, sensory and autonomic ganglia of the peripheral nervous system, a subset of central nervous system nuclei, developing kidney and testis germ cells (Manié et al. 2001; de Graaff et al. 2001). RET null mice display impaired development of superior cervical ganglia and enteric nervous system, kidney agenesis, reduction of thyroid C cells, and impaired spermatogenesis (Manié et al. 2001). Accordingly, individuals with germline loss-of-function mutations of *RET* are affected by intestinal aganglionosis causing congenital megacolon (Hirschsprung disease) (Brooks et al. 2005). *RET* loss-of-function mutations have also been identified in congenital anomalies of kidney and urinary tract (CAKUT), either isolated or in combination with Hirschsprung disease (Jain 2009).

Several genetic alterations convert *RET* into a dominantly transforming oncogene. This review will describe *RET*-derived oncogenes that are associated with different types of human neoplasia (Fig. 1B).

***RET/PTC* IN PAPILLARY THYROID CARCINOMA**

***RET/PTC* Oncogenes**

Papillary thyroid carcinoma (PTC) is the most frequent thyroid cancer and endocrine malignancy overall (Nikiforov and Nikiforova 2011). PTC originates from endodermal-derived thyroid follicular cells and is etiologically associated with exposure to ionizing radiation (Williams 2008). PTC features genetic lesions targeting the RTK-RAS-MAPK pathway. Roughly half of PTC cases display activation of *BRAF* oncogene (most commonly secondary to V600E mutation) and a small proportion of them, mainly belonging to the follicular variant-PTC, carry mutations of *RAS* genes (Xing 2005; Nikiforov and Nikiforova 2011). Although quite uncommon, rearrangements of

the *NTRK1* RTK are found in PTC as well (Greco et al. 2010).

In PTC cases that are negative for *BRAF*, *RAS*, or *NTRK1* mutations, chromosomal rearrangements targeting the long arm of chromosome 10 cause the disruption of *RET* gene and its fusion to various heterologous genes (Grieco et al. 1990). Such chromosomal aberrations give rise to chimeric oncogenes named *RET/PTC* (Fig. 1B). *RET/PTC* oncogenes are composed by the tyrosine kinase and COOH-tail encoding sequence of *RET* (from exon 12 to the 3'-end) and fused at the 5' end to the promoter sequence and 5'-terminal exons of heterologous genes (Fig. 2) (Nikiforov and Nikiforova 2011). The fusion partner genes encode proteins that share the presence of protein-protein interaction domains, such as coiled-coil motifs able to mediate RET TK dimerization (Figs. 1B and 2). Most common *RET/PTC* rearrangements (90% of the cases) are *RET/PTC1* and *RET/PTC3*, the fusions between *RET* and *CCDC6* or *NCOA4* (*RFG*, *ARA70*) genes, respectively. *RET/PTC1* and *RET/PTC3* (and *RET/PTC4*, another *NCOA4-RET* fusion variant) are generated through a paracentric inversion of the long arm of chromosome 10, where *RET*, *CCDC6*, and *NCOA4* map (Grieco et al. 1990; Santoro et al. 1994). Instead, the other *RET/PTC* variants are generated by translocations between different chromosomes and are either rare (*RET/PTC2*) or identified only in single cases of radiation-induced PTC (Fig. 2).

***RET/PTC* Prevalence**

The frequency of *RET/PTC* rearrangements (average 25% of the cases) varies considerably in different patient series (Nikiforov and Nikiforova 2011). This may depend on patients' exposure to different etiologic factors. As an example, in pediatric patients and in cases from areas contaminated by radioiodine isotopes, *RET/PTC* frequency can reach 50%–70% (Zhu et al. 2006). It is also possible that variable prevalence of *RET/PTC* may depend on the methodology used for the detection. Accordingly, the rearrangement can be present only in a subset of cancer or even benign cells (nonclonal *RET/*

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Figure 2. Schematic representation of RET/PTC oncoproteins. On the *top*, wild-type RET protein is illustrated. For each RET/PTC rearrangement, the name of the fusion partner is indicated on the *left* and the corresponding chromosomal alteration is indicated on the *right*. The fusion points are indicated by arrowheads. The length in amino acids of the partner protein portion is also indicated. Boxes in red indicate dimerization (coiled-coil) domains. SP, Signal peptide; TM, transmembrane domain; TK, tyrosine kinase domain; CCDC6, coiled-coil domain-containing protein 6; PRKAR1A, protein kinase, cAMP-dependent, regulatory, type I, alpha; NCOA4 (RFG, ARA70), nuclear coactivator 4 (RET-fused gene, androgen receptor-associated protein 70); RFG5 (GOLGA5), RET-fused gene 5 (Golgin A5); TRIM24, tripartite motif-containing 24; RFG7 (TRIM33), RET-fused gene 7 (tripartite motif-containing 33); KTN1, kinectin 1; RFG9, RET-fused gene 9; ELKS (RAB6IP2), glutamate, leucine, lysine, serine-rich sequence (RAB6-interacting protein 2); PCM1, pericentriolar material 1; RFP (TRIM27), RET finger protein (tripartite motif-containing 27); HOOK3, hook homolog 3.

PTC); and in these cases, it is detected only when highly sensitive techniques (such as nested reverse transcriptase-PCR or real-time reverse transcriptase PCR) or single-cell assays (such as FISH: fluorescent in situ hybridization) are used (Unger et al. 2004; Rhoden et al. 2006; Zhu et al. 2006).

RET/PTC Oncogenic Activity

Adoptive expression of RET/PTC oncogenes induces thyroid cell transformation in vitro

(Santoro et al. 1993; Wang et al. 2003; Melillo et al. 2005). Moreover, targeted expression of RET/PTC in thyroid follicular cells induce thyroid hyperplasia or neoplasia in transgenic mice (Santoro et al. 1996; Powell et al. 1998). This evidence supports the causal contribution of RET/PTC formation to PTC development. Nevertheless, the low penetrance of the disease in transgenic animals, as well as the presence of RET/PTC rearrangements in papillary microcarcinoma, that may not progress to invasive cancer (Viglietto et al. 1995), suggests that ad-

ditional oncogenic events should occur and cooperate with *RET/PTC* to generate an overt disease. Notably, the acute expression of *RET/PTC* in immortalized rat thyroid follicular cells in vitro activates a proapoptotic response because of unscheduled activation of the RAS-MAPK pathway (Castellone et al. 2003; Wang et al. 2003). Moreover, expression of *RET/PTC* in primary human thyrocytes induces oncogene-induced senescence (OIS) (Vizioli et al. 2011). It is not uncommon that normal cells oppose a barrier to neoplastic transformation by switching on suicidal or senescent programs in response to oncogene activation (Hanahan and Weinberg 2011). Thus, it is conceivable that *RET/PTC* rearrangements may be fairly frequent events in thyroid cells but insufficient alone to induce a full-blown cancer, unless further mutational events or epigenetic modifications occur to enable cells to escape cell death or growth arrest defenses.

Mechanisms of *RET/PTC* Formation

Breakage of *RET* and partner genes and their fusion are believed to result from unfaithful repair of DNA double-strand breaks (Ameziane-El-Hassani et al. 2010; Gandhi et al. 2010a). *RET* and its most common fusion partners (*CCDC6* and *NCOA4*) seem to be particularly susceptible to breakage because they map in DNA fragile sites (Gandhi et al. 2010b). Fragile sites are nonrandom DNA loci that are stable under normal conditions but become hot spots of chromosome breakage under exposure to different agents, such as ethanol, caffeine, and hypoxia (Durkin and Glover 2007).

In addition, *RET* gene disruption may be caused by genotoxic agents such as ionizing radiation and reactive oxygen species. *RET/PTCs* are enriched in patients with a known history of exposure to internal (because of thyrocyte ability to concentrate radioiodine) (Williams 2008) or external beam (Collins et al. 2002) radiation, as well as in atomic bomb survivors (Hamatani et al. 2008). Accordingly, *RET/PTC* formation can be experimentally induced by irradiation of cultured thyrocytes and thyroid tissue xenografts in SCID mice (Ito et al. 1993; Mizuno

et al. 1997, 2000; Caudill et al. 2005). However, it is important to note that most patients with *RET/PTC*-positive cancer do not have a documented exposure to radiation. In these cases, genotoxic agents other than radiation may cause DNA breakage (Gandhi et al. 2010b). Of note, H_2O_2 , a potent DNA-damaging agent, is produced in large amounts by thyrocytes during the process of thyroid hormone biosynthesis and may represent one such agent (Ameziane-El-Hassani et al. 2010). Therefore, *RET/PTC*-specific occurrence in thyroid cancer might be explained by the fact that thyroid gland is commonly exposed to agents such as ionizing radiation or H_2O_2 that can disrupt *RET* and its fusion partners. In addition, in thyroid tissue, simultaneous breakage of *RET* and fusion partners, as well as their recombination, may be facilitated by thyroid cell-specific architecture of nuclear chromatin. It has been shown, indeed, that *CCDC6*, *NCOA4*, and *RET* loci display close proximity specifically in thyroid follicular cell chromatin (Nikiforova et al. 2000; Gandhi et al. 2006).

Mechanisms of *RET/PTC* Oncogenic Activation

Two major mechanisms underlie *RET* oncogenic conversion on *RET/PTC* formation. First, secondary to gene fusion, *RET* tyrosine kinase encoding domain is placed under the transcriptional control of the promoter and regulatory elements of *RET* fusion partners. Differently from *RET*, whose expression is restricted to neuroectoderm-derived cells, partner genes are ubiquitously expressed and able to drive *RET* expression in thyroid follicular cells. In addition, fusion to heterologous proteins containing protein homodimerization motifs results in constitutive *RET* kinase dimerization, ligand-independent activation, and autophosphorylation followed by continuous activation of downstream signaling pathways (Fig. 1B) (Bongarzzone et al. 1993; Monaco et al. 2001).

The expression of a constitutively active *RET* kinase leads to chronic exposure of thyroid follicular cells to the activation of intracellular signaling pathways, such as RAS-MAPK, which

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is initiated at the level of RET tyrosine 1062 (Y1062) (Fig. 1A). Intriguingly, this pathway includes BRAF, the other oncogenic protein commonly activated in PTC (Fig. 3). Constitutive signaling is, in turn, responsible for the acquisition of several hallmarks of cancer cells including cell autonomy (independence from growth factors like TSH—thyroid-stimulating hormone), cell motility, and invasion (Melillo et al. 2005). RET/PTC signaling is also able to induce remodeling of the tumor stroma that may facilitate tumor growth. Accordingly, several reports have shown that RET/PTC via the RAS-MAPK cascade endorses an inflammatory-like response characterized by the production of several cytokines and chemokines that, in turn, recruit macrophages, lymphocytes, and mast cells within the tumor thereby promoting cell survival, invasion, and angiogenesis (Russell et al. 2003; Borrello et al. 2005; Melillo et al. 2005, 2010; Puxeddu et al. 2005).

RET Fusion Partners

It is possible that, besides causing activation of RET kinase, *RET/PTC* rearrangements affect also the function of *RET* fusion partners, this in turn contributing to thyroid tumorigenesis. According to this possibility, the rearrangement might cause a genetic double hit, inducing simultaneously the gain of *RET* oncogenic activity and the knockdown of the tumor suppressor function of *RET* partner gene. The *RET/PTC2* rearrangement nicely illustrates this possibility. In this case, the *RET* fusion partner is represented by the *PRKARIA* gene, which encodes the regulatory subunit $R\alpha$ of protein kinase A. *PRKARIA* is a bona fide tumor suppressor gene that is targeted by germline inactivating mutations in patients affected by the Carney complex. This is a rare autosomal dominant cancer syndrome characterized by lentiginosis, atrial, and cutaneous myxoma,

Figure 3. RAS-MAPK oncogenic signaling cascade activated by RET oncoproteins in MTC (left) and PTC (right). In red are indicated other mutational targets (RAS and BRAF) associated with MTC or PTC, respectively. In most of the cases, these mutations are alternative and not present simultaneously in a single tumor.

pituitary adenoma, testicular tumors, ovarian cysts, schwannoma, and thyroid neoplasia (Kirschner et al. 2000; Boikos and Stratakis 2006). Another example may be represented by *TRIM24* (also known as *HTIF1*), the *RET* fusion partner in *RET/PTC6* (Klugbauer and Rabes 1999). *TRIM24* null mice develop hepatic cell carcinoma, and *TRIM24* gene, on chromosome 7q32, shows frequent genetic aberrations in human hepatic cell carcinoma, strongly suggesting its role as a tumor suppressor gene (Wong et al. 1999; Khetchoumian et al. 2007). Finally, several studies also suggest that *CCDC6* and *NCOA4*, the genes involved in most common *RET/PTC* variants (*RET/PTC1* and *RET/PTC3*, respectively), might display tumor suppressor function. *CCDC6* gene product is a ubiquitously expressed 65 kDa protein that displays proapoptotic activity and is involved in ATM-mediated cellular response to DNA damage (Celetti et al. 2004; Merolla et al. 2007). A role of *CCDC6* in the repression of CREB1, a transcriptional factor essential for thyroid cell growth and differentiation, has also been described (Leone et al. 2010). *NCOA4* gene encodes a 70 kDa protein that functions as a coactivator of PPAR γ (peroxisome-proliferator-activated receptor γ) and AR (androgen receptor) (Yeh and Chang 1996; Heinlein et al. 1999). The ectopic overexpression of *NCOA4* in prostate cancer cells reduces cell proliferation and *NCOA4* expression is reduced in aggressive prostate and breast cancers, thus suggesting that this gene may function as a suppressor of tumorigenesis (Kollara et al. 2001; Li et al. 2002; Ligr et al. 2010).

RET MUTATIONS IN MEDULLARY THYROID CARCINOMA AND MEN2 SYNDROMES

Multiple Endocrine Neoplasia Type 2

Medullary thyroid carcinoma (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 adults, around 25% of cases are familial occurring in the frame of inherited cancer syndromes named multiple endocrine neoplasia type 2 (MEN2) syndromes

(online Mendelian inheritance in men, OMIM: #171400) (de Groot et al. 2006). MEN2 comprises three related disorders: MEN2A, MEN2B, and familial medullary thyroid carcinoma (FMTC). MEN2A, first described in 1961 by J.H. Sipple, is characterized by MTC associated with pheochromocytoma (a benign tumor of adrenal medulla) in 50% of cases and parathyroid hyperplasia or adenoma in 10%–30% of cases; more rarely, MEN2A patients show other disease features such as cutaneous lichen amyloidosis and congenital megacolon (see below). In MEN2B syndrome, MTC is associated with pheochromocytoma, ganglioneuromatosis of the intestine, thickening of corneal nerves, and marfanoid habitus (de Groot et al. 2006). Finally, MTC is the only disease phenotype of patients displaying FMTC. Recently, some investigators have suggested FMTC as a phenotypic variant of MEN2A with decreased expression.

RET and MEN2

Specific germline missense mutations of *RET* gene cause the MEN2 syndromes (Fig. 4). Most MEN2A and FMTC mutations affect cysteines in the extracellular cysteine-rich domain of RET. MEN2A is associated most frequently with mutations of cysteine 634 (85%), particularly C634R, whereas FMTC mutations are evenly distributed among the various cysteines (C609, C611, C618, C620, C630) (de Groot et al. 2006). Rare MEN2A or FMTC mutations in RET ectodomain do not target cysteine-rich domain (de Groot et al. 2006; Fazioli et al. 2008; Castellone et al. 2010). FMTC can also be associated with changes in the RET kinase domain (including E768D, L790F, V804L, and V804M).

Most MEN2B patients carry the M918T mutation in RET kinase domain, whereas only a small fraction of them harbor the A883F substitution (Fig. 4). Very rarely, the MEN2B phenotype is sustained by double mutations targeting either the same or two different *RET* alleles (de Groot et al. 2006).

Finally, several additional rare germline *RET* variants have been identified through the systematic screening of MTC patients. However, their pathological significance is not always ob-

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Figure 4. Representation of the most common germline missense mutations in *RET* gene found in MEN2 syndromes. The location of the mutation with respect to RET protein domains and RET exons is indicated. MEN2 phenotypes associated with the various mutations are indicated on the right.

vious, especially if data on cosegregation of the mutation with the disease and functional studies are not available. A comprehensive database annotating all *RET* variants and their pathogenic relevance has been generated (Margraf et al. 2009).

Genetic testing in MTC patients is important not only to distinguish sporadic from familial cases (and thus to identify mutation carriers in the family at risk of developing the disease) but also to assess risk of developing aggressive MTC and MTC-associated neoplastic lesions, such as pheochromocytoma and parathyroid adenoma. Accordingly, most common

RET mutations have been classified to different disease risk levels, ranging from A (less severe) to D (most severe). Risk level classification guides decisions regarding timing of prophylactic thyroidectomy and intraoperative management of the parathyroid glands (Kloos et al. 2009).

RET and Sporadic MTC

MTC arises sporadically in about 75% of cases and *RET* somatic mutations, mainly M918T, occur in about 50% of sporadic MTC, but very rarely in sporadic pheochromocytoma (Beldjord et al. 1995; Lindor et al. 1995). The presence

of somatic *RET* mutation correlates with an aggressive MTC disease phenotype (Romei et al. 1996; Schilling et al. 2001). Recently, sporadic MTC negative for *RET* mutations have been shown to frequently display mutation of *RAS* genes (Moura et al. 2011). Thus, similarly to follicular cell-derived thyroid tumors, thyroid C-cell-derived carcinomas commonly feature the activation of RET-RAS-MAPK pathway (Fig. 3).

MECHANISMS OF *RET* ONCOGENIC CONVERSION SECONDARY TO POINT MUTATIONS

The mechanisms leading to *RET* oncogenic conversion in MEN2 and MTC depend on the site of the amino-acid change. In the case of cysteine mutants, cysteine removal is believed to prevent the formation of intramolecular disulfide bonds, thus allowing free cysteine residues to form intermolecular bonds and mediate the formation of covalent RET dimers with constitutive kinase and signaling activity (Fig. 3) (Santoro et al. 1995). Moreover, differently from wild-type (wt) RET, C634 RET mutant is resistant to intracellular domain cleavage and does not show cytotoxic activity in the absence of ligand, thereby losing the “dependence” receptor feature. This implies that a single mutation (e.g., C634) may induce at the same time increased mitogenic signaling and reduced proapoptotic activity (Bordeaux et al. 2000). Mutations associated with FMTC, which generally target cysteines other than C634 (Fig. 4), are less potently transforming than MEN2A-associated C634 mutations because of their weaker ability to induce formation of RET dimers (Carlomagno et al. 1997; Ito et al. 1997; Chappuis-Flament et al. 1998).

A change in substrate specificity together with a ligand-independent activation of the enzymatic function has been implicated in the mechanism of activation induced by M918T mutation (Santoro et al. 1995; Songyang et al. 1995). In line with this model, M918T mutants differ from wild-type RET in the stoichiometry of phosphorylation of RET tyrosines and of various intracellular proteins (Santoro et al. 1995;

Salvatore et al. 2001). RET/M918T-expressing tumors have different gene expression profiles compared with RET/C634-expressing tumors (Jain et al. 2004). Moreover, X-ray crystallographic analysis of RET tyrosine kinase domain has shown that wild-type RET kinase adopts a head-to-tail autoinhibited dimeric state and that this inactive conformation is destabilized by M918T mutation (Knowles et al. 2006). The mechanism through which RET intracellular mutations (other than M918T) activate constitutively RET enzymatic function has not been clearly elucidated.

RET Gain- and Loss-of-Function in Disease

As mentioned above, germline mutations in *RET* have been implicated in both sporadic and familial cases of Hirschsprung disease (HSCR). HSCR is characterized by the congenital absence of enteric innervation, causing block of peristalsis and bowel obstruction (congenital megacolon) (Brooks et al. 2005). *RET* mutations found in HSCR patients are heterogeneous, ranging from gene deletions to nonsense and missense point mutations, and in most cases they cause a loss of RET signaling. Moreover, some HSCR mutations were found to cause a constitutive caspase-3-mediated RET cleavage and proapoptotic activity probably attributable to the “dependence receptor” features of RET (Bordeaux et al. 2000).

In a few cases, HSCR cosegregates with MEN2A/FMTC that instead, as described above, are associated with RET gain-of-function. These promiscuous HSCR-MEN2A/FMTC cases typically display mutations in RET cysteines other than C634. The most reasonable explanation of this paradox relies on the possibility that these particular mutations have a *Janus*-faced effect, as they feature both a decreased cell surface expression and a constitutive, although low-level, kinase activity. This may on the one hand be sufficient to cause thyroid C-cells transformation, but on the other hand not be sufficient to sustain correct enteric neurons development for the reduced ability of such mutants of interacting with the ligand on the cell surface (Carlo-

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magno et al. 1997; Ito et al. 1997; Chappuis-Flament et al. 1998; Arighi et al. 2004).

RET IN MALIGNANCIES OTHER THAN THYROID CARCINOMA

RET in Lung Cancer

For many years, *RET* oncogenic conversion has been thought to be confined to thyroid cancer. More recently, structural *RET* alterations or changes of its expression have been described in neoplasms affecting organs other than thyroid. Systematic high-throughput sequencing screening identified the M918T *RET* mutation in one single case of non-small-cell lung cancer (NSCLC). In this case, *RET* mutation co-occurred with K-RAS mutation, whereas K-RAS is generally alternative to RTK mutation (Thomas et al. 2007). Very recently, *RET* has been shown to play an important role in a subset of NSCLC patients. In about 1% NSCLC, particularly in adenocarcinoma, chromosomal inversions cause the fusion of the *RET*-encoded TK domain (from exon 12 to the 3'-end of *RET*, as in *RET*/*PTC* rearrangements) to different 5'-terminal exons (15, 16, 22, 23, or 24 exons in different rearrangement variants) of the *KIF5B* (kinesin family member 5B) gene (Ju et al. 2012; Kohno et al. 2012; Li et al. 2012; Lipson et al. 2012; Takeuchi et al. 2012). Less commonly, the *RET*-encoded TK domain was found to be fused to *CCDC6*, *NCOA4*, or *TRIM33* genes (as in *RET*/*PTC1*, *RET*/*PTC3* and *RET*/*PTC7*, re-

spectively) (Li et al. 2012; Wang et al. 2012; Drilon et al. 2013). Similarly to *RET*/*PTC* rearrangements, *KIF5B*-*RET* fusion proteins are likely to form homodimers through the coiled-coil domain present in the NH₂-terminal portion of *KIF5B* (Fig. 5). The coiled-coil domain is retained in all variants of *KIF5B*-*RET* rearranged proteins. Consistently, *KIF5B*-*RET* fusion proteins display ligand-independent activation of *RET* kinase and are able to transform fibroblasts in vitro (Kohno et al. 2012).

RET in Leukemia

RET gene was found up-regulated in a subtype of acute myeloid leukemia with myelomonocytic stage of differentiation (Camos et al. 2006). More recently, gene rearrangements causing the fusion of the *RET*-encoding TK domain (from exon 12 to the 3'-end of *RET*, as in *RET*/*PTC* and *KIF5B*-*RET*) in one case to the first 5'-terminal 4 exons of *BCR* (breakpoint cluster region) and in another case to the first 5'-terminal 12 exons of *FGFR1OP* (fibroblast growth factor receptor 1 oncogenic partner) genes have been cloned from two cases of chronic myelomonocytic leukemia (CMML) (Ballerini et al. 2012). *BCR*-*RET* and *FGFR1OP*-*RET* fusion proteins act as bona fide oncoproteins, display aberrant activation of *RET* kinase, and transform hematopoietic cells in vitro. Thus, although the prevalence of these rearrangements is still unknown, CMML appears to be another neoplasia that,

Figure 5. Schematic representation of *RET* gene rearrangements in human cancer. *RET*/*PTC* rearrangements in PTC are shown, together with *KIF5B*-*RET* (found in lung adenocarcinoma) and *BCR*-*RET* and *FGFR1OP*-*RET* (found in chronic myelomonocytic leukemia). All the *RET* fusion partners invariably code for protein–protein dimerization motifs (depicted as red boxes).

together with PTC and lung adenocarcinoma, is associated with *RET* activation through gene rearrangement (Fig. 5).

RET Role in Additional Cancer Types

Systematic DNA sequencing in a set of colon cancers has revealed the rare occurrence of somatic *RET* sequence variants (Wood et al. 2007). Similarly, sequencing of around 1500 cancer-related genes uncovered the presence of rare *RET* variants of unknown functional significance in hormone receptor positive breast cancer (Kan et al. 2010).

Apart from mutations, many reports have indicated a positive correlation between *RET* overexpression and ER (estrogen receptor)-positive breast carcinoma (Tozlu et al. 2006; Esseghir et al. 2007). *RET* transcriptional promoter was found to display three EREs (estrogen receptor elements) that may mediate *RET* up-regulation by estrogens (Boulay et al. 2008; Kang et al. 2010). A functional link between *RET* and ER is indicated by the observation that *RET* inhibition restored a hormone-sensitive phenotype of antiestrogen-resistant breast cancer cells (Plaza-Menacho et al. 2010).

Some frequent *RET* sequence variants (polymorphisms) have been correlated to specific tumor types. *RET* G691S polymorphism cosegregates with MTC, pancreatic cancer, and desmoplastic subtype of cutaneous malignant melanoma (Robledo et al. 2003; Cebrian et al. 2005; Sawai et al. 2005; Narita et al. 2009). G691S caused increased receptor-signaling responses to GDNF stimulation, with augmented cell proliferation, migration, and invasion (Narita et al. 2009).

RET protein was found overexpressed in pancreatic carcinoma and GDNF polarized migration of pancreatic cancer cells toward nerves mediating neural invasion (Veit et al. 2004; Gil et al. 2010). Finally, although the molecular basis was not elucidated, overactivation of *RET* protein has been observed in glioblastoma multiforme and involved in maintaining a robust downstream signaling able to limit the efficacy of therapies with kinase inhibitors (Stommel et al. 2007).

RET PROTEIN AS AN ANTINEOPLASTIC THERAPEUTIC TARGET

RET Kinase Inhibitors

Small molecule protein kinase inhibitors (PKI) are an important class of anticancer agents. In most of the cases, they compete with ATP, thereby obstructing autophosphorylation and signal transduction downstream from the targeted kinase (Zhang et al. 2009). Prominent examples of successful PKI are represented by imatinib (Gleevec) against BCR-ABL in chronic myeloid leukemia (CML), gefitinib (Iressa), and erlotinib against EGFR in non-small-cell lung carcinoma (NSCLC), and vemurafenib (PLX4032) against BRAF in melanoma (Zhang et al. 2009).

Based on its involvement in cancer and particularly in MTC, a neoplasm that does not respond to conventional chemotherapy, *RET* has raised interest as a molecular target of kinase inhibitors (Schlumberger et al. 2008; Gild et al. 2011). Preclinical studies have shown that MTC cell lines are addicted to *RET* oncogenic signaling and that *RET* PKIs are able to block MTC cell proliferation (Schlumberger et al. 2008). Some compounds were isolated that exerted potent *RET* inhibition. They include vandetanib (ZD6474), sorafenib (BAY 43-9006), sunitinib (SU11248), cabozantinib (XL184), lenvatinib (E7080), and ponatinib (AP24534) (Carlomagno et al. 2002, 2006; Kim et al. 2006; Verbeek et al. 2011; De Falco et al. 2013). These compounds are multitargeted, being able to inhibit *RET* and also additional kinases.

Therapeutic Targeting of *RET* in Human Cancer

Most of *RET* PKIs have been or are being evaluated in clinical trials for MTC treatment (Schlumberger et al. 2008). In particular, vandetanib (ZD6474), an inhibitor of *RET*, EGFR (epidermal growth factor receptor) and VEGFR (vascular endothelial growth factor receptor), and cabozantinib, an inhibitor of *RET*, MET, and VEGFR, have been approved by the FDA for MTC treatment, based on significant progression-free survival prolongation in clinical trials (Kurzrock et al. 2011; Wells et al. 2012).

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However, it should be noted that some specific RET mutations (codons 804 and 806) confer resistance to vandetanib and would require alternative inhibitors (Carlomagno et al. 2004, 2009).

Medullary thyroid cancer (MTC) may not remain the only cancer that benefits from RET kinase inhibition. Papillary thyroid carcinoma (PTC) in general responds well to adjuvant radioiodine treatment and therefore RET kinase inhibitors may not prove particularly useful for this tumor (Gild et al. 2011). Given the recent discovery of RET gain-of-function in lung adenocarcinoma and CMML, it is feasible that RET kinase inhibitors may also find applications in these cancers. Consistently, KIF5B-RET transformed fibroblasts growth was inhibited by vandetanib (ZD6474) (Kohno et al. 2012), treatment with cabozantinib induced objective response in some patients with RET mutant lung adenocarcinoma (Drilon et al. 2013), and treatment with sorafenib (BAY 43-9006), another RET kinase inhibitor, induced cytological, and clinical remission in a patient carrying the BCR-RET fusion (Ballerini et al. 2012).

CONCLUSIONS

After its initial isolation from patients affected by papillary thyroid carcinoma, RET receptor tyrosine kinase also proved to be a key player in the pathogenesis of medullary thyroid carcinoma (MTC). More recently, rare forms of leukemia and non-small-cell lung carcinoma have been associated with oncogenic conversion of RET, thus expanding the spectrum of cancer diseases associated with mutations of this gene. This knowledge will foster efforts to develop agents able to target RET oncoproteins for cancer treatment.

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