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Dysfunction of the RET receptor in human cancer

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Abstract. RET is the receptor for glial-derived neurotrophic factor growth factors. It is a paradigm of a single gene that causes different types of human cancer when targeted by different genetic alterations. Like other receptor tyrosine kinases, once activated, RET recruits a variety of signaling molecules that mediate biological responses. Here we review data on the signaling pathways that lead to RET-mediated cell transformation and recent evidence that manipulation of RET holds promise for thyroid cancer treatment.

Key words. Thyroid; tyrosine kinase inhibitors; RET; MEN2; papillary thyroid carcinoma.

The RET protein

The RET proto-oncogene, located on chromosome 10 q11.2, encodes a receptor tyrosine kinase (RTK). The gene was isolated in 1985 and shown to be activated by a DNA rearrangement (REarranged during Transfection) [1]. The first evidence of RET involvement in human cancer was obtained in thyroid gland papillary carcinomas [2]. RET is a single-pass transmembrane protein (fig. 1). Its extracellular portion contains four cadherin-like repeats, a calcium-binding site and a cysteine-rich domain [3–4]. The intracellular portion features a typical tyrosine kinase domain (fig. 1). RET is expressed primarily in peripheral enteric, sympathetic and sensory neurons, and in central motor, dopamine and noradrenaline neurons. It is also expressed in branching ureteric bud during embryogenesis and in differentiating spermatogonia [5–6]. RET has been detected in human tumors of neural crest origin: neuroblastoma [7], pheochromocytoma and medullary thyroid carcinoma [8] – findings that, together with its chromosomal localization, implicated RET in multiple endocrine neoplasia type 2 (MEN2) syndromes.

Gene ablation studies demonstrated that RET is essential for the development of the sympathetic, parasympathetic and enteric nervous systems (ENS) and the kidney [9]. Excellent reviews of the RET phenotype can be found in Manié et al. [10] and Airaksinen and Saarma [11]. Functional RET disruption by germline mutations causes congenital aganglionosis of the gastrointestinal tract leading to megacolon (Hirschsprung's disease, HSCR; Online Mendelian Inheritance in Men, OMIM 142623) [10]. HSCR, first recognized by the Danish pediatrician Harald Hirschsprung in 1888, has an incidence of 1 in 5000 newborns. It is caused by failure of neural crest-derived ganglion cells to migrate into the intestinal tract thus leading to defective peristalsis and bowel obstruction. At least eight genes are associated with HSCR, none of the mutations being fully penetrant. RET mutations cause ~50% of familial and 15–35% of sporadic HSCR cases. Mutations identified in the RET extracellular domain markedly impair cell surface expression of the protein, probably because of incorrect folding. Mutations in the kinase domain virtually abolish RET enzymatic activity [10]. Finally, a few mutations in the RET carboxyl-terminal tail impair binding to adaptors, i.e., Shc [12], FRS2 [13], IRS1 [14] and ShcC (Rai/N-Shc) [15]. The HSCR phenotype is a complex genetic trait that is influenced by protein activity thresholds and modifier genes. Recently, Fitze and colleagues found that the c135G/A (Ala45Ala) RET polymorphism, perhaps by affecting RET expression levels, modulates the phenotypic effects of RET germline mutations [16].

Also EDNRB, which encodes the seven-pass G-coupled endothelin (ET-1, -2 and -3) receptor, is implicated in HSCR etiology (~5% of cases). ET-3 is essential for formation of the ENS. Genome-wide association studies and mouse models revealed genetic interactions between the RET and EDNRB genes [17–21], which indicated biochemical interactions between the two proteins (fig. 1). Accordingly, Vassilis Pachnis and co-workers showed that

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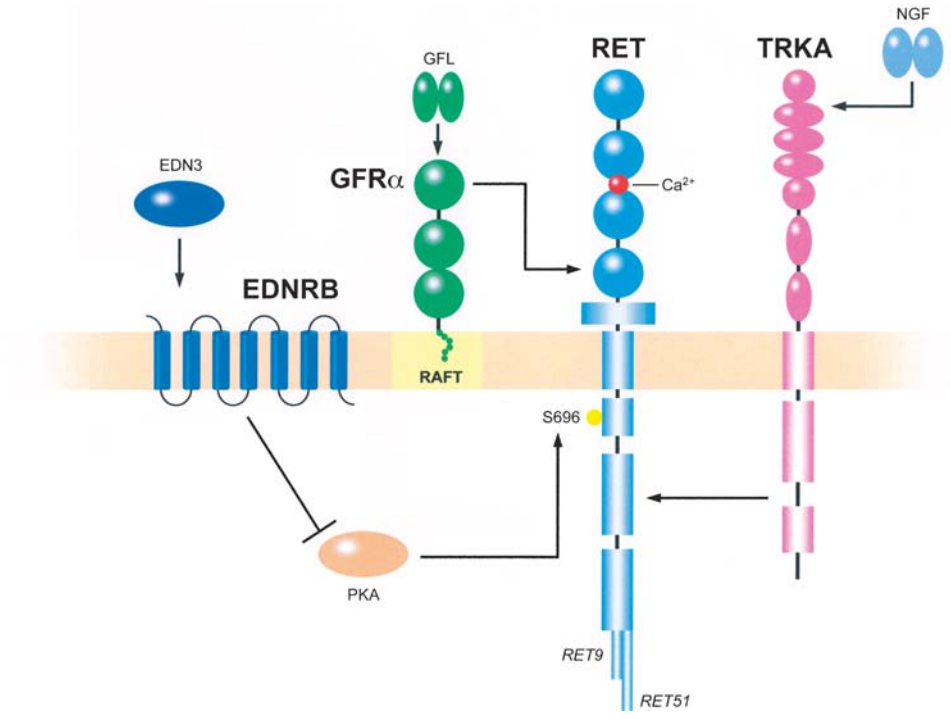

Figure 1. Schematic drawing of the RET protein with the four extracellular cadherin-like domains, the cysteine-rich box adjacent to the plasma membrane, the intracellular juxtamembrane domain containing serine 696 a PKA phosphorylation site and the split tyrosine kinase domain. The two alternatively spliced forms, RET9 and RET51, are indicated. RET is stimulated by complexes of GDNF-family ligands (GFL) with GPI-anchored GFRα co-receptors that are distributed within lipid rafts. The functional interaction of RET with endothelin-3-EDNRB and NGF-TRKA ligand-receptor complexes is shown.

EDNRB activation enhances the effects of RET signaling on the proliferation of uncommitted ENS neurons but inhibits RET-mediated chemoattractive effects [19]. EDNRB reduces cyclic AMP (cAMP) production via $G\alpha_i$, thus leading to protein kinase A (PKA) inhibition. Since chemical PKA blockade mimics the effects exerted by ET-3 on ENS progenitors, it is likely that the RET-EDNRB interaction is mediated by PKA (fig. 1) [19]. This is supported by the finding that serine 686 in the juxtamembrane domain of RET is phosphorylated by PKA, thereby enhancing RET-mediated lamellipodia formation [20]. RET and EDNRB may interact on other levels. Of note, the B-raf serine/threonine kinase, a mediator of neurotrophic factor signaling, is involved in EDNRB-stimulated MAPK activation [22] and it appears to be also a key mediator of RET (see below).

RET stimulation mediated by GDNF ligands

RET participates in a cell-surface protein complex that binds growth factors of the glial-derived neurotrophic factor (GDNF) family (fig. 1). Four GDNF family ligands (GFLs) have been isolated: GDNF, neurturin (NRTN), artemin (ARTN) and persephin (PSPN). GDNF was identified in 1993 as a survival factor for midbrain dopaminergic neurons [23], and soon raised expectations as a potential therapeutic agent for Parkinson's disease [24]. GFLs support several neuronal populations including enteric neurons. GDNF also regulates kidney and spermatogonial differentiation [10-11]. GFLs bind RET in conjunction with glycosylphosphatidylinositol (GPI)-anchored co-receptors designated GDNF family receptor-α (GFR-α). Each of the four GFLs uses one of the four GFRs-α (GFRs-α 1–4) as preferential receptor [11]. GFLs form a high-affinity complex with the corresponding GFR-α homodimer, and the complex brings together two RET molecules, thereby triggering autophosphorylation and intracellular signaling (fig. 1). Some RET molecules may be weakly bound to GFR-α even before GFL binding [11]. The GPI anchor localizes GFR-α to lipid rafts. GDNF binding to GFR-α recruits RET to lipid rafts, thereby facilitating RET interaction with the c-Src kinase and the FRS2 docking protein. Outside the rafts, activated RET mainly binds to Shc. It is noteworthy that FRS2 and Shc bind to the same phosphotyrosine in RET (Y1062, see below). Therefore, activation by GDNF will lead not only to RET activation but also to binding to a restricted set of intracellular signal transducers [11]. Importantly, RET oncoproteins carrying MEN 2A mutations mainly reside outside rafts, suggesting that oncogenic conversion of RET may cause aberrant signaling outside rafts [25].

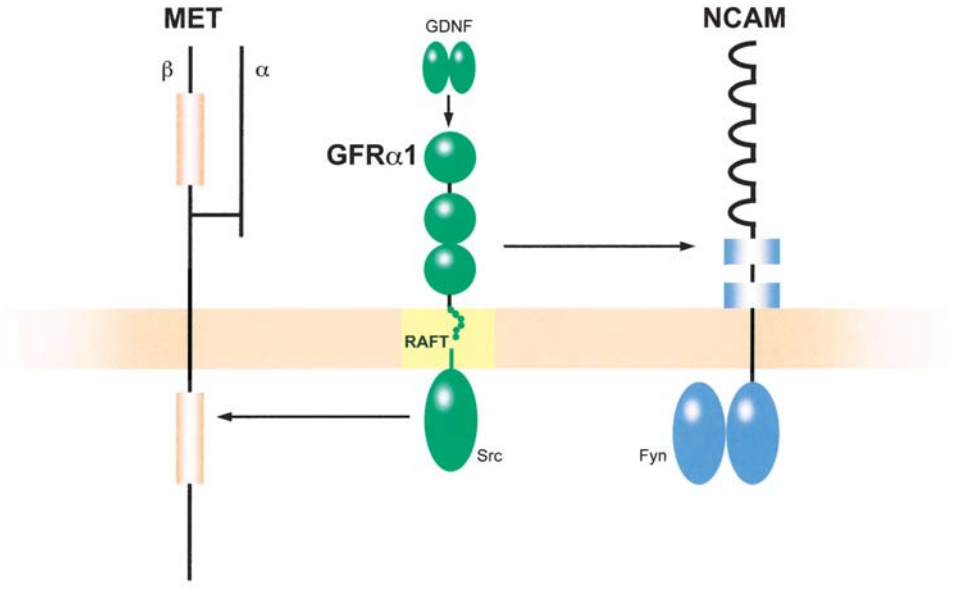

Figure 2. RET-independent signaling of GDNF. The GDNF- GFRα1 complex is able to trigger NCAM signaling through the c-Src family kinase Fyn, and to trigger c-Src activation that in turn provokes trans-phosphorylation of Met, the HGF receptor.

GDNF signaling needs heparan sulphate glycosaminoglycans, such as syndecans and glypicans, probably to ensure local concentration of GDNF [26]. GDNF is able to trigger intracellular signaling events that are mediated by GFR-α but not by RET [27]. Stimulated GFR-α can trigger c-Src activation; in turn, c-Src induces phosphorylation of Met, the tyrosine kinase receptor of hepatocyte growth factor (HGF) [28]. Thus, Met may contribute to RET-independent GDNF signaling (fig. 2). Carlos Ibanez and collaborators demonstrated that neural cell adhesion molecule (NCAM) functions as an alternative signaling receptor for GFLs (fig. 2). In the presence of GFR-α, GDNF binds with high affinity to p140-NCAM and intracellularly activates the Src-like kinase c-Fyn and the focal adhesion kinase FAK [29].

Intriguingly, RET can be activated independently of the GFL-GFR-α complexes. Nerve growth factor (NGF)-triggered activation of the TrkA kinase receptor promotes RET phosphorylation through GFL-independent inter-receptor kinase signaling (fig. 1) [30]. This TrkA-RET interaction selectively involves RET51, one of the two RET alternative protein products (see below). Of note, both RET and TrkA, the two kinases involved in this developmental interaction, are also involved in the pathogenesis of papillary carcinomas of the thyroid gland [31].

Cell signaling mediated by RET

Autophosphorylated tyrosines in RET have been identified by both phosphopeptide mapping [32] and mass spectrometry [33]. RET contains at least 12 autophosphorylation sites: tyrosine 687 (Y687), Y806, Y809, Y826, Y900, Y905, Y981, Y1015, Y1029, Y1062, Y1090 and Y1096 (fig. 3). Molecular modeling of the RET kinase [33] showed that Y687 maps in the juxtamembrane region, Y806 and 809 in the N-terminal lobe, Y826 in the kinase insert, Y900 and 905 in the activation loop, Y981 in the C-terminal lobe, and Y1015, 1029 and 1062 in the carboxyl-terminal tail. The remaining residues (Y1090 and Y1096) are present only in the RET51 isoform (fig. 3). In addition, Y752 and Y928 have been implicated in RET docking to signal transducer and activator of transcription 3 (STAT3) [34]. Studies with phosphospecific antibodies demonstrated phosphorylation of some of these sites (Y905, Y981, Y1015, Y1062 and Y1096) in intact cells [35–38].

Tyrosine 905, equivalent to tyrosine 416 in c-Src, is the autocatalytic residue, whose phosphorylation probably serves as a local switch to induce a conformational change that activates the kinase [39]. Phosphorylation of Y900 in the activation loop, and of Y806 and 809 in the contralateral side of the activation loop, supplements the function of Y905 [33]. Other RET-phosphorylated tyrosines serve as docking sites for signaling proteins. These interactions are reviewed in Manié et al. [10] and Ichihara et al. [40]. Deletion of Y687 enhances RET induction of lamellipodia formation, suggesting that Y687 recruits protein/s that negatively modulate activation of the Rac small GTPase. In this way, Y687 phosphorylation counteracts the effects of S686 phosphorylation by PKA [20]. Tyrosine 905 is a binding site for Grb7/10 adaptors, Y1015 for phospholipase Cγ, and Y1096, unique to RET51, for Grb2. Tyrosine 981 is a docking site for c-Src [38, 41]. Tyrosine 1062 is the binding site for several proteins including Shc, ShcC, IRS1/2, FRS2, DOK1/4/5 and Enigma. In turn, binding

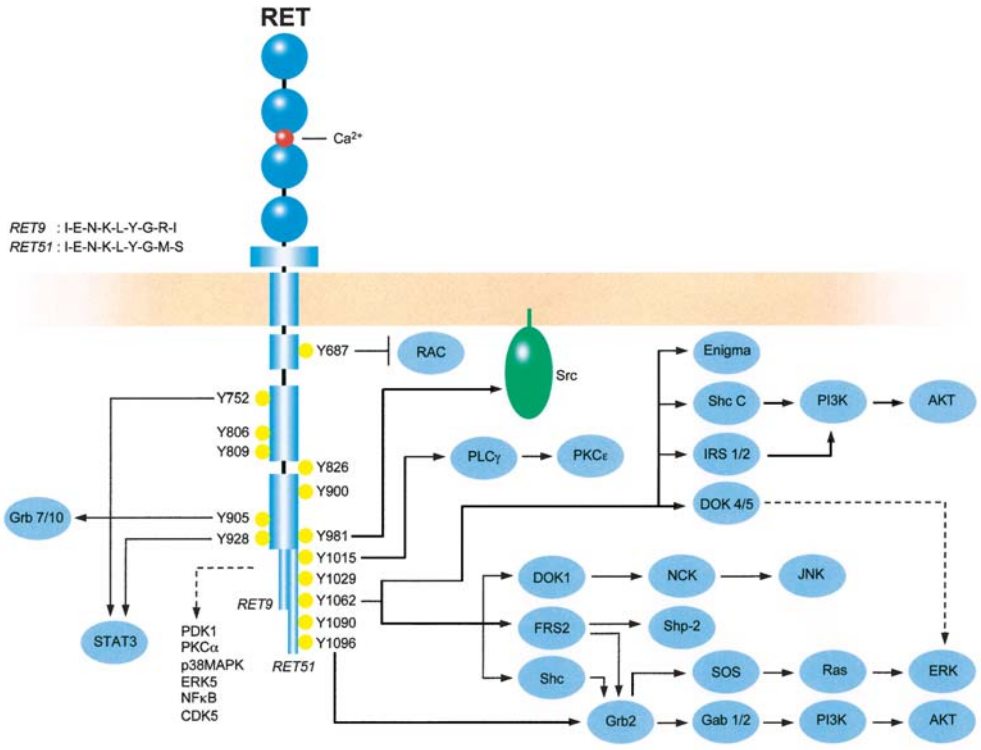

Figure 3. The network of RET-mediated signaling events. RET auto-phosphorylation sites are shown with their direct targets. Dotted lines indicate pathways not yet fully elucidated. The amino acid sequences of RET9 and RET51 at the point in which they start to diverge at glycine 1063 are shown. The NKLY(1062) sequence shared by both isoforms is a good consensus for the binding of PTB domains.

to Shc and FRS2 mediates recruitment of Grb2-SOS complexes leading to Ras/ERK stimulation, and of Grb2-GAB1/2 complexes leading to stimulation of the phosphatidylinositol-3-kinase (PI3K)/AKT pathway. ShcC and IRS are required to trigger PI3K/AKT activation. Binding to DOK4 and DOK5 is implicated in ERK stimulation, whereas binding to DOK1 is involved in recruiting the NCK adaptor leading to c-Jun N-terminal kinase (JNK) stimulation [10, 40] (fig. 3). Enigma is endowed with LIM and PDZ domains and is involved in shuttling rearranged RET/PTC oncoproteins to the membrane (see below). RET stimulation triggers many other signaling intermediates, including CREB [42], p38MAPK [42], NFκB [43], ERK5 (BMK1) [44], phosphoinositide-dependent kinase 1 (PDK1) [45], cyclin-dependent kinase 5 (CDK5) [46] and PKC isoforms [47–48] (fig. 3). Although the mechanism of activation of these pathways remains to be elucidated, Y1062 is important for most of them. In particular, Ras/ERK- and PI3K-triggering by Y1062 is essential for full activation of CREB and NFκB [42]. A novel RET interactor designated Grap-2 has been found to negatively regulate NFκB activation [43].

Thus, RET appears to trigger a complex network of intracellular signaling pathways, with Y1062 being central to most of them. Y1062 is essential for the transforming ability of RET-derived oncogenes in cell cultures [49] and in transgenic animals [50]. Some of these signals could be functionally redundant. Recent insights into the genetic lesions underlying thyroid cancer may help elucidate this network by indicating which pathways are required for the oncogenic effects exerted by RET (see below). The remarkable flexibility of RET Y1062, which is able to bind several different proteins, probably in a mutually exclusive fashion, suggests a model whereby competition between various signaling intermediates for binding to a single docking site may be used to switch between different biological responses to RET-dependent signaling.

RET is subjected to alternative splicing, resulting in two major protein isoforms of 1072 and 1114 amino acids (RET9 and RET 51, respectively). RET9 and RET51 differ in the amino acid sequences immediately downstream from Y1062: starting from glycine 1063, RET9 has 9 extra residues, whereas RET51 features a stretch of 51 amino acids that includes two phosphorylation sites, Y1090 and Y1096 (fig. 3). Vassilis Pachnis and colleagues generated monoisoformic mice for RET9 or RET51. RET9 mice, lacking RET51, were phenotypically normal, whereas RET51 mice had a phenotype similar, albeit milder, to RET-null animals. This demonstrated that RET9 and RET51 have different signaling abilities and that only RET9 is crucial for normal development [51]. Surprisingly, however, oncogenic RET mutations are more potently transforming when inserted in RET51 than in RET9 [49, 52]. There are biochemical differences between RET9

and RET51: (i) RET51 binds less strongly to the SH2 domain of Shc with respect to RET9 [53]; (ii) RET9 and RET51 did not associate with each other [54]; (iii) Y905 and Y1062 phosphorylation is greater in RET9 than in RET51, whereas Y1015 phosphorylation is greater in RET51 [54]; (iv) Enigma binds more strongly to RET9 than to RET51 [55].

RET and neuroendocrine tumors

Germline point mutations in RET cause three related dominantly inherited cancer syndromes: multiple endocrine neoplasia type 2A (MEN 2A), 2B (MEN 2B) and familial medullary thyroid carcinoma (FMTC) (Online Mendelian Inheritance in Men, OMIM 171400). MEN 2 patients are affected by medullary thyroid carcinoma (MTC), a malignant tumor arising from calcitonin-secreting C cells of the thyroid gland. Additional features can be present in MEN 2A (pheochromocytoma in ~50% of cases, parathyroid hyperplasia in ~15–30%, and, more rarely, hereditary localized pruritus) and MEN 2B (pheochromocytoma in ~50% of cases, and more rarely, ganglioneuromatosis of the intestine, thickening of corneal nerves and marfanoid habitus) [56–57]. In MEN 2B patients, MTC is clinically aggressive and starts earlier. FMTC patients usually develop MTC at a later stage in life.

In 1993, Bruce Ponder and co-workers demonstrated that MEN 2A was caused by germline mutations in RET [58]. Soon after it became clear that over 90% of MEN 2 patients carry germline point-mutations in RET, and that the disease phenotype is strongly correlated with specific RET mutations [59]. Most MEN 2B patients carry M918T in the RET kinase domain, whereas only a small fraction of them harbor the A883F substitution (fig. 4). Most MEN 2A and FMTC mutations affect one cysteine (609, 611, 618, 620, 630, 634) in the extracellular cysteine-rich domain of RET (fig. 4). MEN 2A is associated most frequently with mutations of codon 634 (85%), particularly C634R, whereas FMTC mutations are evenly distributed among the various cysteines. To date no FMTC family has been found to carry the C634R mutation. FMTC is also associated to changes in the N-terminal (E768D, L790F, Y791F, V804L, V804M) and C-terminal (S891A) lobe of the RET kinase (fig. 4). Duplications/insertions and deletions in RET have also been reported, albeit more rarely [57]. Somatic mutations of V804, M918 and E768 are found also in sporadic MTC. Other rare germline or somatic mutations, single or in combination, have been found at codons 533, 766, 778, 806, 844, 904, 919, 922 and 930 [57] (fig. 4).

MEN 2-associated RET mutations have a gain-of-function effect, i. e., they promote activation of the kinase and oncogenic conversion. Indeed, MEN 2 was the first example of an inherited cancer syndrome caused by the germline transmission of a dominantly activated oncogene [60]. This has obvious therapeutic implications. The observations of germline mutations in one allele and somatic mu-

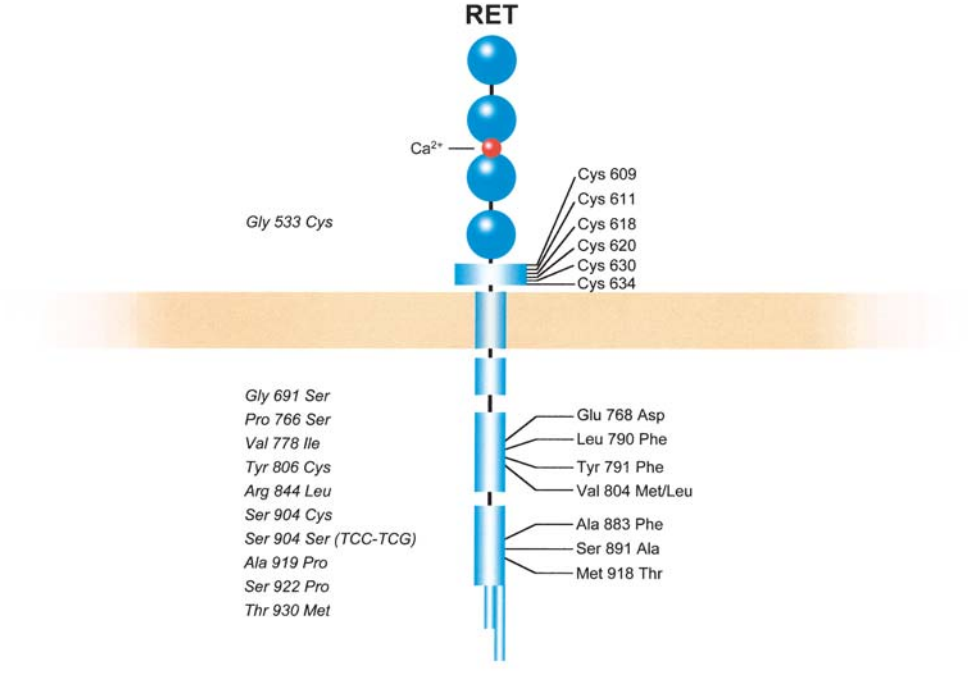

Figure 4. RET protein with the most common mutations in MEN 2 syndromes. Other more rare sequence changes found in rare families or in sporadic MTC cases alone or in combination as well as the Gly691Ser and the TCC(904)-TCG polymorphisms that modify the penetrance of disease are shown in italics on the left.

tations in the other, and loss of the wild-type allele and/or amplification of the mutant RET indicate that imbalance of the mutant and wild-type RET alleles may favor tumor development [61]. Polymorphic RET variants (G691S and S904S) have a modifier effect on the age at which MEN 2A starts. Although the mechanism by which these polymorphisms affect disease phenotype is unknown, it is possible that they affect RET expression levels or, when associated with an amino acid change, the activity of the protein [62]. Mechanisms leading to RET oncogenic conversion in MEN 2 depend on the location of the amino acid change. Extracellular cysteine mutants display constitutive kinase activity consequent to disulfide bonds stabilized homodimerization [60, 63]. These cysteine residues are thought to be normally involved in the formation of intramolecular disulfide bonds. Thus, when a cysteine is mutated, a partner cysteine may become free and form an aberrant intermolecular bond between two mutated RET monomers. Cysteine mutations in the extracellular domain of RET sometimes induce the FMTC phenotype. More than 60% of these FMTC mutations target cysteines other than 634, which, instead, is more often associated with MEN 2A. FMTC-associated mutations are less potently transforming than MEN 2A-associated mutations. Indeed, mutations at cysteine 634 have a stronger transforming ability than mutations targeting the other cysteines. The low transforming ability of the latter reflects the marked reduction of mutant RET protein on the cell surface [64]. This model would also explain why MTC co-segregates with mutations in codons 609, 611, 618 or 620 in 20–30% of families. These mutations may cause constitutive RET activation to a level that is probably sufficient to initiate C-cell transformation. However, by diminishing the level of RET protein at the plasma membrane, they can also impair responsiveness to GDNF and cause defective intestinal innervation [65].

Little is known of the mechanisms of RET activation by mutations targeting the intracellular domain. These mutations probably modify the structure of the kinase by switching on its enzymatic function. The crystal structure of the RET kinase will solve this issue. A change in substrate specificity has been implicated in the case of the M918T MEN 2B mutation, which maps in the P+1 loop of the kinase [60, 66]. In line with this model, MEN 2B mutants display differences in the stoichiometry of autophosphorylation of the various RET tyrosines with respect to MEN 2A mutants, i.e., Y1062 phosphorylation is enhanced [36] and Y1096 phosphorylation is reduced [32]. Furthermore, RET-MEN 2B is more potent than RET-MEN 2A in phosphorylating the DOK1 adaptor [67] and a panel of proteins, including paxillin, which bind CRK and NCK adaptors [68]. Consistent with this peculiar signaling, MEN 2B-expressing cells have different gene expression profiles with respect to MEN 2A-expressing cells [69].

RET and papillary thyroid carcinoma

Papillary thyroid carcinoma (PTC) is the most prevalent thyroid malignancy, accounting for 80–90% of thyroid

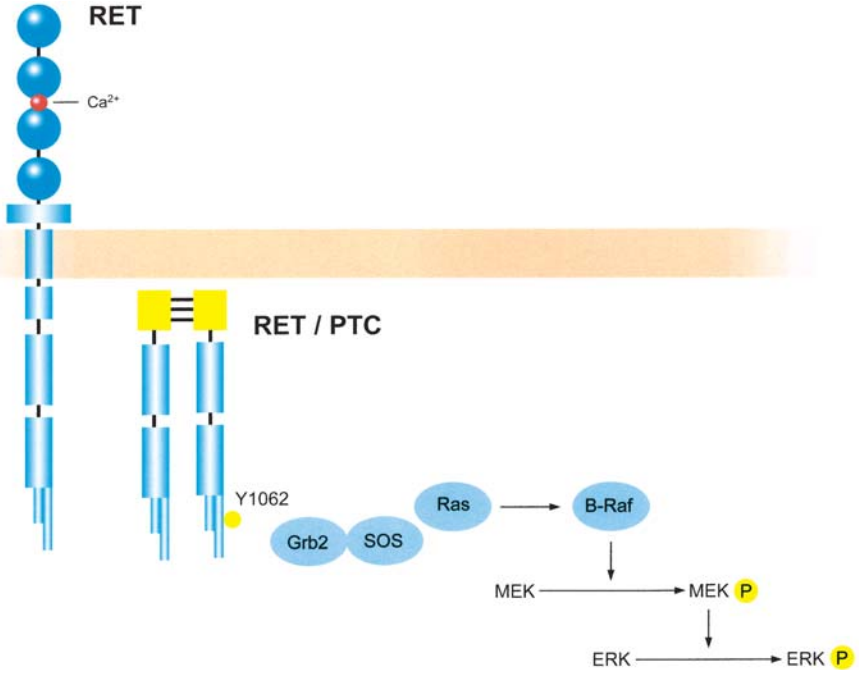

Figure 5. RET/PTC rearrangements in PTC. The hypothetical signal transduction cascade that starts from RET/PTC and leads to ERK triggering through B-raf is represented.

cancers. The hallmarks of PTC are chromosomal inversions or translocations of 10q11.2, which occur in 2.5–40% of cases. These chromosomal aberrations cause the recombination of the intracellular kinase-encoding domain of RET with heterologous genes, thereby generating the RET/PTC oncogenes (fig. 5). Interestingly, the NGF-receptor TrkA, is similarly rearranged in a small PTC subset [31].

In PTCs, RET is rearranged with diverse genes: H4/D10S170 (leading to RET/PTC1); the regulatory subunit Iα of the cAMP-dependent protein kinase A (RIα; in RET/PTC2); the RET fused gene (RFG), also designated EleI/ARA70/Ncoa4 (in RET/PTC3 and 4); RFG5/golgin-84 (in RET/PTC5); human transcriptional intermediary factor 1 (HTIF1; in RET/PTC6); RFG7/HTIFγ (in RET/PTC7); KTN1/kinectin (in RET/PTC8); RFG9 (in RET/PTC9); ELKS (glutamic, leucine, lysine and serine-rich protein) (in ELKS-RET); pericentriolar material (PCM1; in PCM1-RET) and RET finger protein (RFP; in RFP-RET) [70]. Intriguingly, RFP-RET, recently isolated from a PTC in an externally irradiated patient [71], is almost identical with the rearrangement in RET isolated by Takahashi and colleagues [1]. RET/PTC1 and 3 (fig. 5) are the most prevalent variants.

A body of evidence supports the notion that RET/PTC oncogenes can be causative in thyroid tumorigenesis. RET/PTC transforms thyroid follicular cells in vitro [72] and induces the irregular nuclear contours and euchromasia that are hallmarks of PTC [73]. RET/PTC-transgenic mice develop PTC [70]. A high proportion of occult microscopic PTC foci, thought to be precursors of fully manifest PTC, display RET/PTC rearrangements, suggesting that RET/PTC may be an early genetic change in PTC development [74].

The mechanism of RET oncogenic activation consequent to RET/PTC rearrangements is not fully understood. While RET expression is normally very restricted, its fusion partners are ubiquitously expressed. Therefore, RET/PTC rearrangements may lead to the unscheduled expression of the RET tyrosine kinase in thyroid cells. However, RET can be expressed in follicular cell-derived tumors even in the absence of rearrangement [75]. Virtually all the translocated amino termini fused to RET are predicted to fold into coiled coils. Thus, fusion with such protein-protein interaction motifs provides RET/PTC kinases with dimerizing interfaces. In addition, RET/PTC recombinations delete the extracellular, transmembrane and intracellular juxtamembrane domains. In this context, we recently observed that the juxtamembrane domain suppresses RET mitogenic signaling [76]. RET/PTC oncoproteins are expected to be relocated to the cytoplasm. This could prevent RET/PTC interacting with signaling intermediates distributed at the plasma membrane and allow phosphorylation of unusual cytosolic substrates. In this context, by interacting with proteins resident at the plasma membrane, like Enigma [77] and FRS2 [13], RET/PTC proteins could be redirected to the membrane compartment. Finally, an intriguing possibility is that a functional alteration of the RET fusion partners consequent to the rearrangement may contribute to carcinogenesis. Although verification of this awaits characterization of the normal function of the various RET fusion partners, it is noteworthy that H4 (the RET partner in RET/PTC1) is a pro-apoptotic protein [78] and RFG (the RET partner in RET/PTC3) is a transcriptional co-activator of the peroxysome proliferator activated receptor-gamma PPARγ steroid hormone receptor that also exerts pro-apoptotic effects in thyroid cells [79]. Finally, RIα (the RET partner in RET/PTC2) is a tumor suppressor gene that causes the Carney complex, a multiple neoplasia syndrome that consists of endocrine and non-endocrine tumors [80].

Ionizing radiations promote double-strand DNA breaks and thyroid cancer is associated with radiation exposure [81]. Therefore, a direct link between radiations, RET/PTC rearrangements and PTC has been envisaged. In support of this hypothesis, in vitro and in vivo irradiation induces fusion between the H4 and RET genes [82]. After the meltdown of the Chernobyl reactor (26 April, 1986), the incidence of childhood PTC increased up to 100-fold in Belarus, Ukraine, and the western regions of Russia [81]. RET/PTC rearrangements have been found in over 60% of post-Chernobyl PTCs. Recently, Yuri Nikiforof and co-workers proposed an intriguing mechanism to explain how ionizing radiation can cause RET/PTC rearrangements and why this occurs preferentially in thyrocytes. They showed that, while RET and H4 loci are about 30 megabases apart in the linear map of chromosome 10, they frequently juxtapose in nuclei of thyroid cells but not in other cell types. This spatial contiguity provides the structural basis for radiation-induced illegitimate nonhomologous recombination of the two genes [83]. Another model was proposed to explain the peculiar susceptibility of thyroid follicular cells to chromosomal rearrangements. In vitro exposure of thyroid cells to ionizing radiation did not induce apoptosis, but significantly increased DNA end-joining enzymatic activity. Thus, thyrocytes may respond to DNA damage with chromosomal alterations rather than cell death [84].

Molecular genetic studies revealed another genetic feature that may shed light on RET-mediated mechanisms of tumorigenesis. Activating point mutations in B-raf were found in ~45% of cases of PTC [85]. All the B-raf mutations identified thus far affect nucleotide 1796 in exon 15, and result in a thymine-to-adenine transversion, which translates into valine-to-glutamate substitution at residue 599 (V599E). B-raf belongs to the RAF family of serine/threonine kinases and is located downstream of RAS small GTPases and upstream of MEK in the classic MAPK cascade. Importantly, there was no overlap between PTC cases harboring B-raf and those with RET/

PTC mutation. This implies that in thyroid cells, B-raf and RET/PTC act along the same epistatic signaling cascade (fig. 5). It is conceivable that B-raf is a key mediator also of wild-type RET.

RET as a target for therapeutic intervention

Like monoclonal antibodies against the extracellular domain of oncogenic receptors, small molecule tyrosine kinase inhibitors are an important new class of anti-cancer agents. Such inhibitors compete with ATP, but are sufficiently specific to be devoid of substantial side effects. We recently identified three powerful RET inhibitors: two pyrazolo-pyrimidines: 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]-pyrimidine (PP1) and 4-amino-5-(4-chloro-phenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine) (PP2), and an anilino-quinazoline: N-(4-bromo-2-fluorophenyl)-6-methoxy-7-[(1-methylpiperidin-4-yl)-methoxy]quinazolin-4-amine (ZD6474) [86–88]. These compounds showed half-maximal RET inhibitory concentrations in the nanomolar range (≤100 nM). When injected into nude mice, fibroblasts transfected with oncogenic RET lost morphological transformation, proliferative autonomy, anchorage-independent growth and tumorigenicity. In addition to inhibition of RET phosphorylation, PP1 induces RET destruction through proteosomal degradation [89]. ZD6474 in particular holds promise for the treatment of RET-related thyroid tumors. It has pharmacokinetics compatible with once daily oral administration, and a phase I trial revealed no significant toxicity [90]. ZD6474 is also a potent inhibitor of KDR, the vascular endothelial growth factor (VEGF) receptor, and thus exerts anti-angiogenic effects [90]. The arylidene 2-indolinone RPI-1 was effective against RET but only at high doses (IC_{50} in the micromolar range) [91]. Two indolocarbazole derivatives, CEP-701 and CEP-751, inhibit RET-MEN 2A oncoproteins at concentrations <100 nM. Importantly, these compounds also inhibited tumor growth in MTC cell xenografts [92]. Resistance to kinase inhibitors may occur because of the selection of clones with mutations in residues important for binding to the drug. Thus, one may envisage that cocktails of different chemical classes of inhibitors would overcome resistance in the case of RET-targeted therapeutic intervention.

Concluding remarks

RET was first isolated in 1985. In less than 3 decades, researchers discovered its role in papillary thyroid carcinomas and MEN 2 syndromes, in congenital megacolon and in development. Soon after the discovery that RET was the gene responsible for MEN 2, genetic testing to treat patients with early thyroidectomy was described [56]. As such, MEN 2 is probably the first successful example of the use of genetic testing to treat a familial cancer. We envisage that in a relatively short time small organic compounds that block RET tyrosine phosphorylation will be used to treat established RET-positive cancers and to formulate cancer prevention strategies in patients carrying germline RET mutants.

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