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RET as a Diagnostic and Therapeutic Target in Sporadic and Hereditary Endocrine Tumors

Jan Willem B. de Groot, Thera P. Links, John T. M. Plukker, Cornelis J. M. Lips, and Robert M. W. Hofstra

Departments of Endocrinology (J.W.B.d.G., T.P.L.), Surgical Oncology (J.W.B.d.G., J.T.M.P.), and Genetics (R.M.W.H.), University Medical Center Groningen, University of Groningen, 9700 RB Groningen, The Netherlands; and Department of Endocrinology (C.J.M.L.), University Medical Center Utrecht, 3508 GA Utrecht, The Netherlands

The *RET* gene encodes a receptor tyrosine kinase that is expressed in neural crest-derived cell lineages. The RET receptor plays a crucial role in regulating cell proliferation, migration, differentiation, and survival through embryogenesis. Activating mutations in RET lead to the development of several inherited and noninherited diseases. Germline point mutations are found in the cancer syndromes multiple endocrine neoplasia (MEN) type 2, including MEN 2A and 2B, and familial medullary thyroid carcinoma. These syndromes are autosomal dominantly inherited. The identification of mutations associated with these syndromes has led to genetic testing to identify patients at risk for MEN 2 and familial medullary thyroid carcinoma and subsequent implementation of prophylactic thyroidectomy in mutation carriers. In addition, more than 10 somatic rearrangements of *RET* have been identified from papillary thyroid carcinomas. These mutations, as those found in MEN 2, induce oncogenic activation of the RET tyrosine kinase domain via different mechanisms, making RET an excellent candidate for the design of molecular targeted therapy. Recently, various kinds of therapeutic approaches, such as tyrosine kinase inhibition, gene therapy with dominant negative RET mutants, monoclonal antibodies against oncogene products, and nuclease-resistant aptamers that recognize and inhibit RET have been developed. The use of these strategies in preclinical models has provided evidence that RET is indeed a potential target for selective cancer therapy. However, a clinically useful therapeutic option for treating patients with *RET*-associated cancer is still not available. (*Endocrine Reviews* 27: 535–560, 2006)

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Abbreviations: AKT, v-akt murine thymoma viral oncogene homolog 1; CCH, C cell hyperplasia; DOK, downstream of kinase; FMTC, familial MTC; GDNF, glial cell line-derived neurotrophic factor; GFL, GDNF family of ligands; GFRα, GDNF-family α receptors; Grb, growth factor receptor-bound protein; HSCR, Hirschsprung disease; MEN, multiple endocrine neoplasia; MTC, medullary thyroid carcinoma; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; PI3K, phosphatidylinositol 3-kinase; PLC, phospholipase C; PTC, papillary thyroid carcinoma; RAF, Ras effectors serine/threonine kinase; RET, rearranged during transfection; RNAi, RNA interference; RPI-1, ribose-5-phosphate isomerase; sGFRα, soluble GFRα; SHANK3, SH3 and multiple ankyrin repeat domains 3; Shc, Src-homology collagen; SNP, single nucleotide polymorphism; Src, v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog; STAT3, signal transducer and activator of transcription 3; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

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I. Introduction

TYROSINE KINASES ARE involved in the most essential processes of cells, such as the cell cycle, proliferation, differentiation, motility, and survival (1). In several human cancers, key tyrosine kinases are no longer sufficiently controlled. Many tyrosine kinases are constitutively phosphorylated because of tumor-initiating mutations leading to constitutively active signaling proteins (1, 2).

The *RET* gene encodes a receptor tyrosine kinase (RET) that is mainly expressed in precursor cells of the neural crest and urogenital tract. RET is essential for the early development of the sympathetic, parasympathetic, and enteric nervous systems, the kidney, and spermatogenesis (3, 4). Accordingly, inactivating germline *RET* mutations are found to be responsible for the development Hirschsprung disease (HSCR), a congenital absence of enteric neurons in the gastrointestinal tract (5, 6). On the other hand, activating *RET* mutations and rearrangements cause human cancers and cancer syndromes, such as familial medullary thyroid carcinoma (FMTC), multiple endocrine neoplasia (MEN) type 2, and papillary and Hürthle cell thyroid cancer (7–9).

In this review, we will describe the structure and signaling properties of wild-type and mutant RET and its role in human endocrine cancers. Furthermore, we will review the timing of intervention based on genotype and the role of RET as a therapeutic target.

II. The *RET* Gene and Protein

The *RET* gene was first identified in 1985 by transfection of NIH3T3 cells with human lymphoma DNA. The transformed NIH3T3 cells proved to harbor a fusion gene, which was absent in the original tumor. This fusion gene contained part of a gene that encoded a tyrosine kinase domain, and that gene from which the tyrosine kinase domain was part was thereafter called "*RE*arranged during *T*ransfection" (10). *RET* is localized on 10q11.2, is approximately 55,000 bp in size, and contains 21 exons (11).

RET is a single-pass transmembrane protein. It contains four Ca^{2+}-dependent cell adhesion (cadherin)-like domains (to induce and stabilize conformational changes needed for interaction with the ligands and coreceptors) and a juxtamembrane cysteine-rich region (responsible for the tertiary structure and formation of dimers) in the extracellular domain (5, 12). The extracellular domain also contains a number of glycosylation sites (13). The fully glycosylated protein of 170 kDa (also called the mature form of RET) is present on the cell membrane. The immature form of 150 kDa lacks glycosylation and is present only in the endoplasmic reticulum and in the cytoplasm (14). The intracellular region encompasses two tyrosine kinase subdomains (TK1 and TK2) that are involved in the activation of numerous intracellular signal transduction pathways (Fig. 1).

RET is subject to alternative splicing of the 3′ region generating three protein isoforms that contain 9 (RET9), 43 (RET43) and 51 (RET51) amino acids in the carboxy-terminal tail downstream from glycine 1063 (15). RET9 and RET51, consisting of 1072 and 1114 amino acids, respectively, are the main isoforms *in vivo* (Fig. 1).

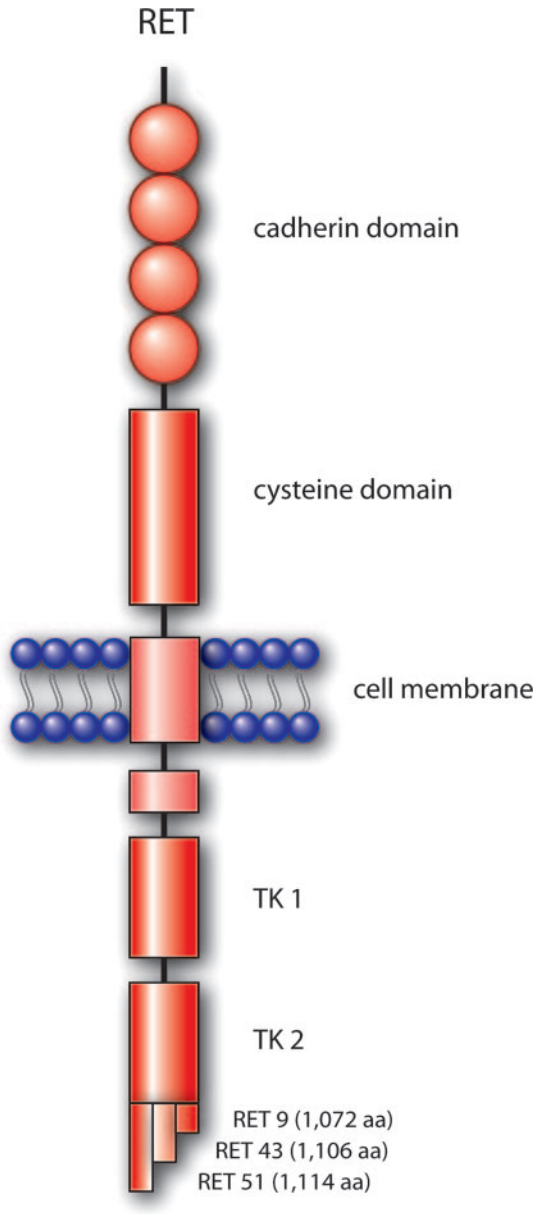

FIG. 1. Schematic representation of the RET tyrosine kinase. The extracellular region comprises four cadherin domains and a cysteine-rich domain. A single transmembrane region spans the cell membrane, and the two tyrosine kinase domains (TK1 and TK2) are located in the intracellular region. The three isoforms of RET are indicated. aa, Amino acids.

III. RET Activation Mediated by Ligands

A. RET as receptor for the glial cell line-derived neurotrophic factor (GDNF) family of ligands

Under normal conditions, RET can be activated by a complex of coreceptors and ligands. These belong to two groups of proteins: the GDNF family of ligands (GFLs), including neurturin, artemin, and persephin; and the glycosylphosphatidylinositol-anchored GDNF-family α receptors (GFRαs) (Fig. 2). One of the four GFLs binds to one of the GFRαs (GFRα1–4) to form a GFRα/GFL complex. GDNF uses GFRα-1 as preferential receptor, neurturin uses GFRα-2, artemin uses GFRα-3, and persephin uses GFRα-4, although there is some cross-specificity (16). Interaction of this GFRα/GFL complex with RET leads to autophosphorylation of tyrosine residues.

Although they are usually bound to the plasma membrane, GFRαs also occur in a soluble form (17). Therefore, RET activation can take place in two ways: in *cis* and in *trans*

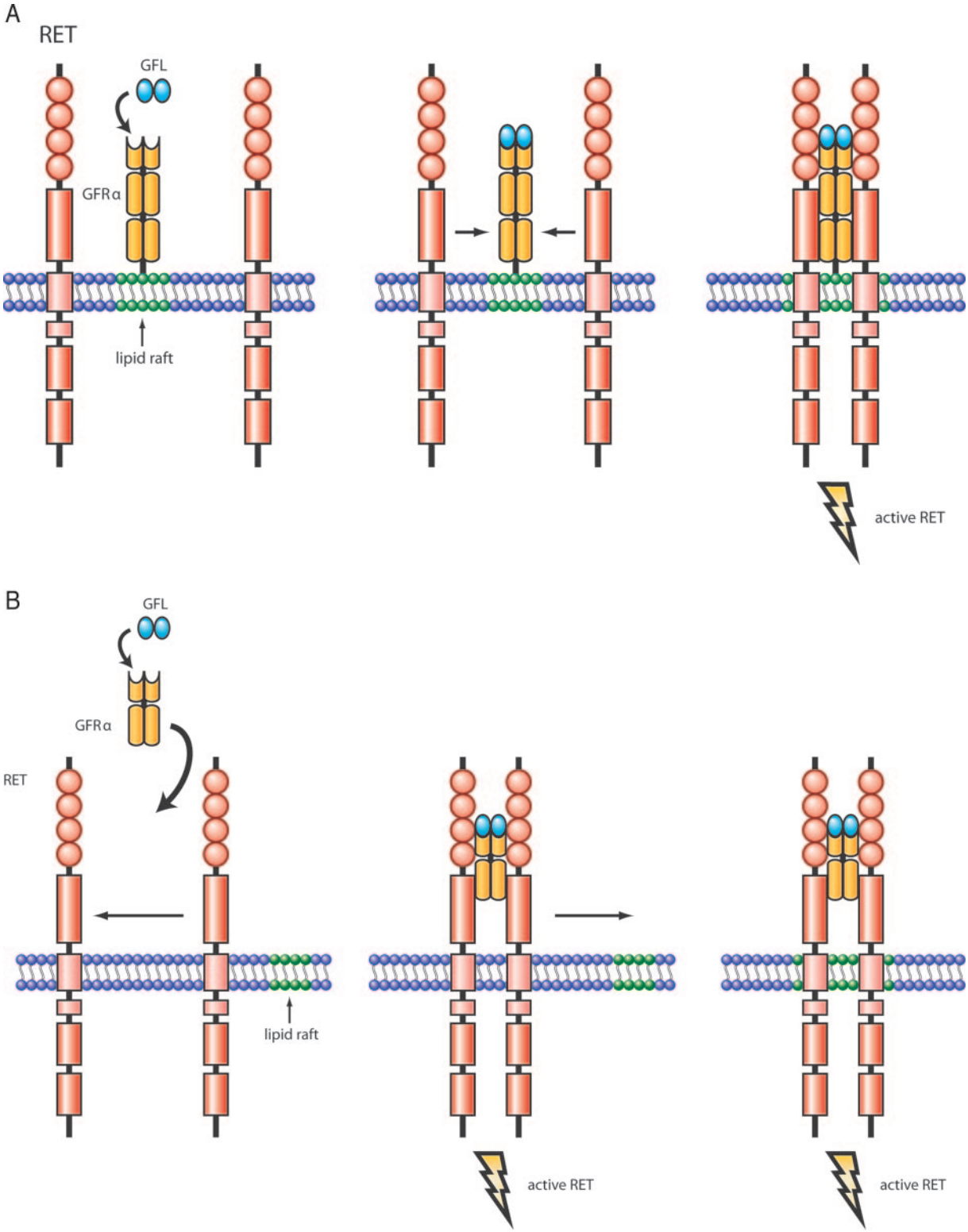

FIG. 2. Different mechanisms of ligand-mediated RET activation. A, RET is activated in *cis* when one of the GDNF family of ligands binds to membrane-bound glycosylphosphatidylinositol-anchored GDNF-family α coreceptors (GFRα) that are distributed within lipid rafts. Activation of RET leads to dimerization of RET, which consequently signals to the nucleus. B, RET activation in *trans*: the ligand binds to the soluble form of its coreceptor (sGFRα) and the ligand-sGFRα complex brings together two inactive RET monomers, initially located outside lipid rafts. Then, the activated RET dimer is recruited to the lipid raft.

(Fig. 2). The *cis* model for RET activation hypothesizes a stepwise assembly of the GFL-receptor complex. The GFL binds to membrane bound GFRα, and subsequently, the GFRα/GFL complex brings together two RET molecules resulting in phosphorylation of tyrosines and intracellular signaling (5, 18–20) (Fig. 2A). The *trans* model of RET activation suggests that the GFL may also bind to the soluble (nonmembrane bound) form of the GFRα coreceptors (sGFRα). The GFL-sGFRα complex then triggers RET activation via dimerization (21, 22).

Membrane-bound GFRαs are known to be located within detergent-insoluble cholesterol-rich domains within the lipid bilayer of the cell membrane, called lipid rafts, which are enriched with signaling proteins (22, 23). These lipid rafts serve as essential signaling compartments in GDNF-stimulated RET signaling and are responsible for cell adhesion and different neuronal processes (21, 24–26).

In its inactive form, RET is located outside the lipid rafts. Upon *cis*-activation, inactive RET is recruited to the lipid rafts by the GFL-GFRα complex (Fig. 2A) and becomes active when associated in the complex within these lipid rafts. This mechanism of activation occurs predominately in cells coexpressing RET and GFRα (24). Because sGFRα is not located within lipid rafts, upon *trans*-activation, RET is already active before it is relocalized to lipid rafts (Fig. 2B). This relocalization process is slower and more persistent and, remarkably, dependent on the activated state of RET, whereas recruitment of RET to lipid rafts in *cis* is independent of the activation status (21, 22).

It is still poorly known whether other GFLs besides GDNF activate RET both in *cis* and *trans*, but it is likely that the other coreceptors (GFRα2–4) differ from GFRα1 regarding the interaction with cell surface proteins (27). All GFRαs induce the phosphorylation of the same tyrosines on the intracellular kinase domains (see *Section IV*) (28), but they do have specific expression patterns, suggesting that each GFRα has distinct roles in RET activation (19).

B. RET activation by other growth factors

Growth factors and their receptors are engaged in a complex network of signals that promote cell growth and differentiation. Although RET is mainly activated by GFLs, other growth factors can activate RET as well. For instance, binding of neurotrophic growth factor to its receptor tyrosine kinase (NTRK1) modulates the phosphorylation of RET51 (and not RET9 or RET43) via an interreceptor kinase signaling mechanism independently of ligands or coreceptors (29), resulting in augmented growth, metabolism, and gene expression.

IV. RET Signaling

A. RET docking sites

RET plays a central role in several intracellular signaling cascades that regulate cellular survival, differentiation, proliferation, migration, and chemotaxis. These pathways are initiated upon RET activation. Specific tyrosine residues, which serve as docking sites for adaptor proteins that link the signal from the receptor to the main signal transduction pathways, are activated through phosphorylation. At least 18 of these specific phosphorylation sites have been identified, including tyrosine 687 (Y687), serine 696 (S696), Y752, Y791, Y806, Y809, Y826, Y864, Y900, Y905, Y928, Y952, Y981, Y1015, Y1029, Y1062, Y1090, and Y1096. RET9 has only 16 tyrosines in the intracellular domain, whereas Y1090 and Y1096 are present only in the long RET 51 isoform (6, 30–32).

B. Signal transduction pathways

A synopsis of signal transduction pathways that are triggered by RET is given below (and in Fig. 3). The pathways triggered by phosphorylation of the different docking sites mentioned above are described below.

GDNF-induced phosphorylation at serine 696 in RET is required for activation of guanine nucleotide exchange factor and lamellipodia formation. Y687 appeared to induce opposite effects on lamellipodia formation. These effects on cytoskeletal rearrangement by activation of RET are regulated via a cAMP/protein kinase A-dependent mechanism (33).

Signal transducer and activator of transcription 3 (STAT3) is a latent transcription factor implicated in several types of cancer when aberrantly activated and an important target of RET through phosphorylation of Y752 and Y928 (34–36).

Y905 interacts with the growth factor receptor-bound protein (Grb) docking proteins 7/10 upon phosphorylation. Phosphorylation of Y905 facilitates autophosphorylation of tyrosine residues located in the C-terminal tail by stabilizing the active conformation of the kinase (37). Y900, Y806, and Y809 probably supplement the function of Y905 (38). The function of the Grb7/10 pathway, however, needs to be further elucidated.

Phosphorylated Y981 constitutes the major binding site of v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (Src) and therefore the primary residue responsible for Src activation upon RET engagement. Activation of Src is essential to neuronal survival (39). However, it also plays a role in oncogenic RET signaling, and Src is a likely candidate to mediate signaling between RET and focal adhesion kinase (40), an important regulator of tumor formation and cell migration, which is required for the invasion and metastasis of cancer cells (41).

Tyrosine 1015 is a binding site for phospholipase C (PLC)-γ, which activates protein kinase C (PKC) enzymes. PKC enzymes, in turn, cause RET phosphorylation but also down-regulate RET and its downstream signaling, thus functioning as a negative feedback loop to modulate RET activity (42). However, when RET activation is prolonged, the PKC-mediated negative feedback loop is down-regulated, leading to cell survival and clonal expansion (43). Furthermore, PLC-γ triggers the release of Ca^{2+} from intracellular stores via the generation of inositol tris-phosphate (44). Although binding of the RET ligands (45) and RET transport to the cell membrane (46) are dependent on Ca^{2+}, the precise effects of RET-induced Ca^{2+} influx are not clear yet.

Phosphorylation of Y1062 is crucial for activation of major intracellular signaling pathways, and ablation of Y1062 leads to a considerable decrease in the transforming activity of RET (47). Y1062 is a docking site for various adaptor proteins,

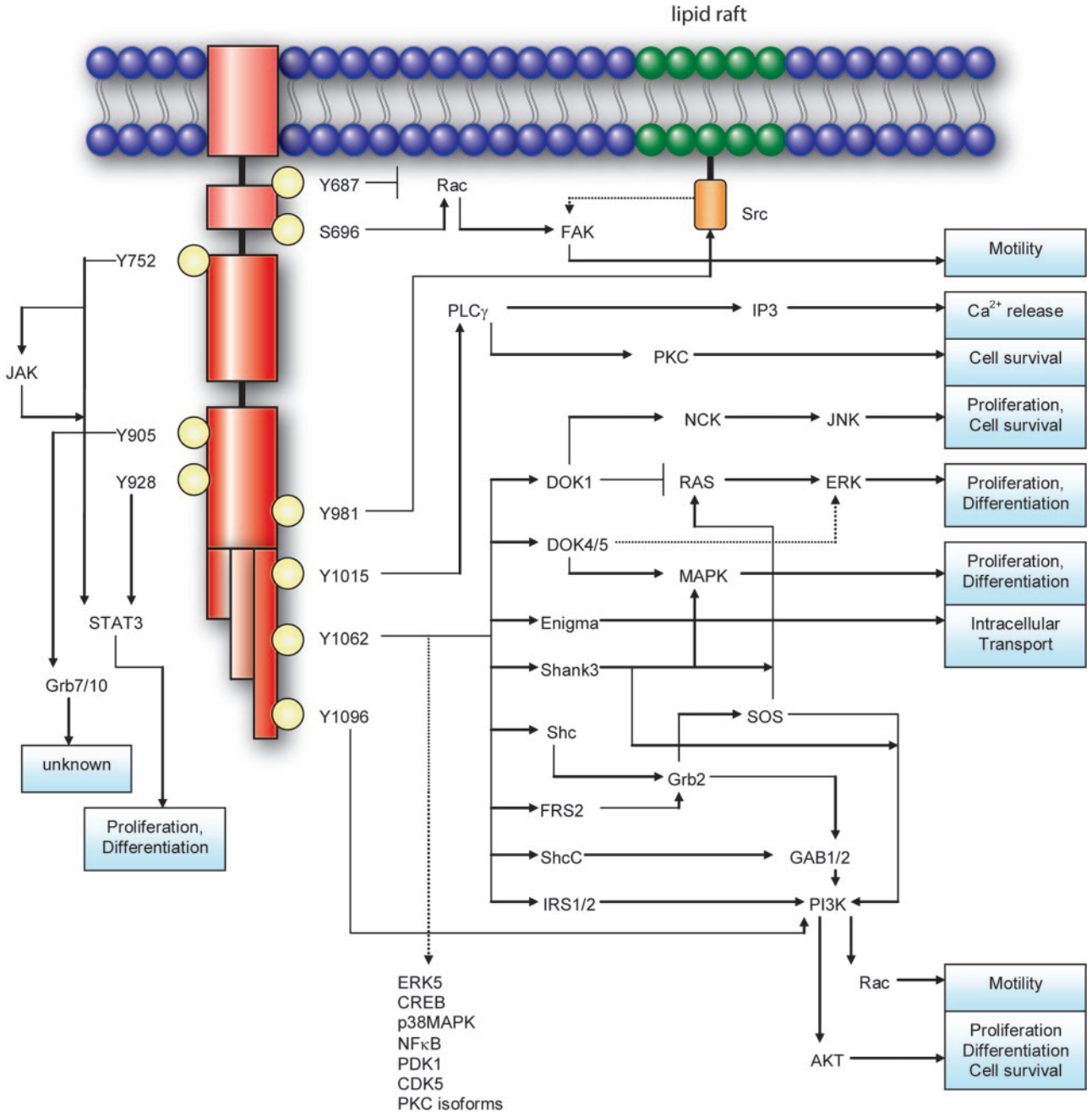

FIG. 3. Synopsis of the signaling network mediated by RET. The docking sites with their direct targets are shown. For the sake of simplicity the lipid raft is depicted outside RET. *Dotted lines* designate pathways that are not completely elucidated.

including Src-homology collagen (Shc), ShcC (also called Rai), insulin receptor substrate 1/2, fibroblast growth factor receptor substrate 2 (FRS2), downstream of kinase (DOK) 1/4/5, Enigma, ERK5, MAPK, phosphoinositide-dependent kinase 1, cyclin-dependent kinase 5 (CDK5), SH3 and multiple ankyrin repeat domains 3 (SHANK3), and PKC isoforms. Shc recruits the Grb2/son of sevenless multi-protein scaffold (SOS) complex and Grb2-associated binding protein (GAB)1/2 resulting in the activation of the phosphatidylinositol 3-kinase (PI3K)/v-akt murine thymoma viral oncogene homolog 1 (AKT) pathway (48). This pathway is responsible for survival signaling, enhanced cell-cycle progression, and RET-mediated transformation (48, 49). The Grb2/GAB complex can also assemble directly onto phosphorylated Y1096, offering an alternative route to PI3K activation by GDNF (50). Recently, it was demonstrated that upon ligand activation, RET was down-regulated and disappeared from the cell surface via ubiquitin-proteasome protein degradation. This phenomenon was mediated by a Shc-Grb2 route, which could be activated through Y1062 and Y1096 (51). These findings demonstrate once more that various negative regulatory pathways closely regulate RET activity.

ShcC, a neuron-specific adaptor protein, and insulin re-

ceptor substrate 1/2 are also required for PI3K/AKT activation (52, 53). Moreover, cell motility and morphology are regulated via PI3K and members of the Rho family of GTPases, including Rho, Rac, and Cdc42 (33, 54, 55). The PI3K/AKT pathway and also the RAS/ERK pathway are important for activation of the transcription factors cAMP response element-binding protein and nuclear factor κB (56). In addition, the binding of Shc as well as FRS2 to the Grb2/SOS complex induces the RAS/ERK and MAPK pathways (57, 58). These pathways contribute to cellular differentiation and proliferation through mitogenic signaling (59). Binding of DOK1 to Y1062 links RET to the Jun N-terminal kinase pathway, which is important in cell proliferation, cell survival, cell death, DNA repair, and metabolism (60, 61) and can suppress the RAS/ERK pathway by RAS-GTPase activating proteins (GAP) (62). DOK4 and DOK5 seem to have opposite effects to DOK1 by triggering MAPK and the ERK pathway (63). Enigma and SHANK3 bind specifically to Y1062 of RET9, despite its phosphorylation state. SHANK3 mediates sustained RAS/ERK, MAPK, and PI3K/AKT signaling (64), and Enigma is involved in transporting rearranged RET oncoproteins to the cell membrane (31, 65). How binding of ERK5 (66), cAMP response element-binding protein (56), p38 MAPK (56), nuclear factor κB (67), phosphoinositide-dependent kinase 1 (68), CDK5 (69), and PKC isoforms (43, 70) to Y1062 functions in the complex network of RET-induced intracellular signaling pathways is not well established.

Finally, Y791, Y826, Y864, Y952, Y1029, and Y1090 are also phosphorylated, but their downstream signaling pathways still need to be delineated (30, 38).

V. The Role of RET during Development and in Human Diseases

In *Sections II, III*, and *IV*, the structure of RET, the various ways of receptor activation, and the diverse RET signaling pathways have been described. Next, the role of RET during development and in endocrine tumors and cancer syndromes will be highlighted.

A. The role of RET during development

RET is expressed mostly in the developing nervous and urogenital systems and plays a crucial role in the development of the enteric nervous system, the kidney, and spermatogenesis (3, 4, 72). In adult tissue, high levels of *RET* were observed in brain, thymus, peripheral enteric, sympathetic and sensory neurons, and testis (3, 6, 73).

At very early stages of development, *RET* is expressed in a cranial population of neural crest cells. A subset of *RET*-positive cells is subsequently observed in central nervous system nuclei, including the motor and catecholaminergic neurons. During development, *RET*-expressing neural crest cells migrate caudally via the intestinal mesenchyme to form the enteric nervous system, located in the gut wall of the gastrointestinal tract (3). Another portion of *RET*-expressing cells gives rise to early development of sensory and autonomic ganglia of the peripheral nervous system, adrenal chromaffin cells, thyroid C cells, and the kidney (for review, see Refs. 6 and 73).

The critical role of *RET* during development is illustrated by the observation that mice expressing null mutations in *RET* lack superior cervical ganglia and the entire enteric nervous system; have agenesis or dysgenesis of the kidney, impaired spermatogenesis, and fewer thyroid C cells; and die shortly after birth (6, 72). The two isoforms *in vivo* of RET behave differently as concluded from *in vitro* assays in which RET 51 showed the highest transforming and kinase activity (74). Several observations suggested that the different isoforms of RET have different tissue-specific effects during embryogenesis. RET9 is sufficient to support normal embryogenesis and postnatal life. Mice expressing only RET51, however, have severe defects in the innervation of the gut and renal development (75).

B. RET and endocrine tumors

1. RET *and papillary thyroid carcinoma (PTC).* The clinical relevance of *RET* in human diseases was first recognized in PTC. PTC is the most prevalent thyroid cancer, accounting for 80 to 90% of all thyroid malignancies (76). There are several somatic genetic lesions associated with PTC, including oncogenic activation of the *RAS* (77), *BRAF* (78), *MET* (79), *TSH-R*, *Gsa*, and *p53* genes (80) and chromosomal alterations that affect *NTRK1* and *RET* (81). Specific rearranged forms of *RET* were detected in PTC (82). These chromosomal aberrations occur in 2.5 to 40% of cases and are the result of double-stranded DNA breaks (mostly radiation-induced), which lead to erroneous reparative fusion of the coding region for the C terminus of RET to the promoter and coding region of the N terminus of a constitutively expressed unrelated gene by virtue of their physical proximity (83). These fusion genes encode proteins that harbor the intracellular kinase domain of RET and the N-terminal domain of various proteins. The N-terminal domains of these various proteins all have the property to let the fusion protein dimerize, leading to autophosphorylation of tyrosine residues in the tyrosine kinase domain of RET. Almost exclusively, the breakpoints in RET occur at sites distributed across intron 11 (84), giving rise to proteins without a transmembrane domain. These gene fusions encode constitutively active cytoplasmic chimeric proteins named RET/PTC.

To date, 12 different fusion partner genes, depicted in Fig. 4, are reported to form (because of variable breakpoints) at least 17 different *RET* hybrid oncogenes (85, 86). The most prevalent variants of these chimeric oncogenes are *RET/PTC1* (60 to 70%) and *RET/PTC3* (20 to 30%) (86–88).

Exposure to external radiation, the major risk factor for the development of PTC, is associated with the formation of *RET/PTC* (83, 89, 90). After the nuclear power plant disaster in Chernobyl on 26 April 1986, the incidence of childhood PTC in Ukraine, Belarus, and neighboring counties increased dramatically in the subsequent years (91, 92) and *RET/PTC* rearrangements have been found in over 60% of post-Chernobyl PTCs (93). Furthermore, a high prevalence of *RET/PTC* has been detected in PTC patients previously subjected to external irradiation for benign or malignant disease (94). Most *RET/PTC* rearrangements are associated with exposure

Chimeric oncoprotein	Fusion partner
RET/PTC1	H4/D10S170
RET/PTC1L	
LongRET/PTC1	
RET/PTC2	PKA R1α
RET/PTC3	RFG
RET/PTC3r2	(ELE1/ARA70/Ncoa4)
RET/PTC3r3	
RET/PTC4	RFG
	(ELE1/ARA70/Ncoa4)
RET/PTC5	RFG5/golgin-84
RET/PTC6	HTIF1
RET/PTC7	RFG7/HTIFγ
RET/PTC8	KTN1/Kinectin
RET/PTC9	RFG9
ELKS/RET	ELKS
PCM1/RET	PCM1
RFP/RET	RFP
ΔRFP/RET	

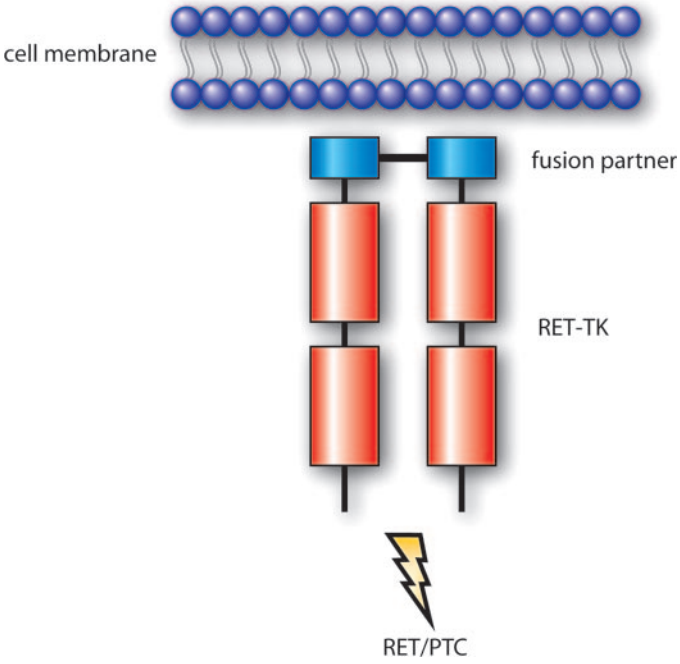

FIG. 4. In papillary thyroid cancer (PTC), rearrangement with various activating genes leads to the formation of chimeric RET oncoproteins. As a result, ligand-independent dimerization of the intracellular tyrosine kinase motifs occurs, leading to constitutive signaling of the RET tyrosine kinase. The diverse gene partners that have been found to rearrange with RET are listed.

to external radiation. Only *RET/PTC1*, *RET/PTC1L*, *RET/PTC2*, *RET/PTC3*, and *ELKS/RET* have been found in non-radiation-associated PTC (85).

Besides the association with ionizing radiation, there are several other indications that point to *RET/PTC* as a causative factor in the pathogenesis of PTC. *RET/PTC* transforms thyroid follicular cells *in vitro* (95), and specific overexpression of *RET/PTC1* and *RET/PTC3* in the thyroid of transgenic mice leads to the development of tumors that resemble PTC (96–98). Interestingly, not all transgenic mice developed thyroid tumors, implying that the expression of the oncoprotein is necessary but not sufficient for tumorigenesis. Conversely, wide differences in the prevalence of *RET/PTC* rearrangements in human PTC have been reported, ranging from 5 to 67% (85, 99, 100). Clearly tumorigenesis involves, besides the RET/PTCs, multiple other genetic lesions in the development of PTC.

In microscopic PTC, *RET/PTC* expression is highly prevalent (99). This suggests that *RET/PTC* is activated at early stages of the disease.

Although several reports failed to demonstrate correlation of *RET/PTC* rearrangements with clinicopathological features of increased morbidity (101–103), different types of *RET/PTC* rearrangement are associated with variation in biological behavior. Patients with *RET/PTC1* usually show an indolent behavior, whereas *RET/PTC3* is associated with a more aggressive tumor phenotype (104–107). These observations are in keeping with transgenic mouse models expressing *RET/PTC*. Mice harboring *RET/PTC1* develop thyroid lesions with morphological features of PTC that do not metastasize, whereas mice carrying *RET/PTC3* are associated with solid tumor growth and metastases (97, 98).

Although *RET/PTC* rearrangements have been observed in Hashimoto's thyroiditis (108, 109), the absence of *RET/PTC* in PTC arising in the background of Hashimoto's thyroiditis suggests that the molecular basis of the association of Hashimoto's thyroiditis with follicular-derived thyroid cancer is different from *RET/PTC* rearrangement (110).

Somatic rearrangements of *RET* have also been found in familial PTC, which is more aggressive than its sporadic counterpart (111, 112). However, because linkage between *RET* and the disease phenotype is excluded, *RET* is not a predispositionary factor in familial PTC (113).

2. RET and Hürthle cell carcinoma. The heterogeneous group of Hürthle cell neoplasms of the thyroid gland has been a matter of ongoing controversy regarding the histological classification, assessment of clinical behavior, and treatment recommendations (114, 115). Hürthle cell carcinomas are considered by some to be oxyphilic variants of follicular thyroid cancer (116), but others consider them a distinct histopathological entity (115). Hürthle cell tumors of the thyroid are unusual neoplasms characterized by the presence of oncocytes, which are large polygonal cells with hyperchromatic, often bizarre, nuclei and an eosinophilic granular cytoplasm. Most Hürthle cell carcinomas do not take up radioiodine and are generally believed to be more aggressive than follicular thyroid cancers (114, 115).

Several studies confirmed that *RET/PTC* is not restricted to PTC but can also occur in Hürthle cell adenomas and carcinomas (9, 100, 117, 118). Hyperplastic nodules with oncocytic metaplasia are generally negative for *RET/PTC* activation (117). *RET/PTC* activation can probably be considered a secondary event in Hürthle cell adenomas and carcinomas, subsequent to the occurrence of genetic alterations determining oncocytic metaplasia. Remarkably, Hürthle cell adenomas and carcinomas showed a comparable rate of *RET/PTC* rearrangements (117). Therefore, one could consider Hürthle cell tumors always malignant, much like PTCs, which actually share the same genetic variation. This may explain why the distinction between benign and malignant Hürthle cell tumors is very difficult and why apparently benign tumors at histological examination may give rise to distant metastasis. This may also explain why Hürthle cell carcinomas are thought to be more aggressive, simply be-

cause only the most aggressive forms are currently considered malignant.

3. Oncogenic RET activation in PTC and Hürthle cell carcinoma. In the absence of rearrangements, *RET* expression is very restricted (but not absent) in thyroid follicular epithelial cell-derived tumors (119). The genes fused with *RET*, however, are constitutively expressed within thyroid follicular cells, and *RET/PTC* rearrangements therefore allow constitutive expression of the kinase domain of *RET*, which is essential for the malignant transformation of the thyroid cells (120). In addition, fusion with protein partners holding protein-protein interaction motifs provide RET/PTC kinases with dimerizing lineages, which results in ligand-independent autophosphorylation (31). Furthermore, RET/PTC recombinations delete the transmembrane domains that suppress mitogenic signaling (121), and hence it is likely that these oncoproteins are relocated to the cytosolic compartment of the cell. For that reason, another important function of the proteins that are rearranged with RET is in determining a localization at the plasma membrane, although interaction of RET/PTC with Enigma may be responsible for this relocalization process as well (31, 65). The various activating fusion partners of RET may be distributed in different cellular compartments, permitting RET to interact with diverse groups of signaling proteins. This may be an explanation for the variation in oncogenic potential between different *RET*-associated types of PTC (87).

To obtain more insight in oncogenic RET signaling caused by rearrangements, it should be emphasized that RET/PTC signaling depends mainly on three key docking sites: Y905, whose phosphorylation stabilizes the active conformation of the kinase domain (37); Y1015, whose prolonged phosphorylation down-regulates a PKC-dependent negative feedback loop to promote cell survival and clonal expansion (43); and Y1062, whose phosphorylation recruits numerous signal transduction proteins to RET/PTC (6).

The oncogenic proteins involved in the initiation of PTC generally work along the same linear signaling cascade. Phosphorylation of tyrosine 1062 is relevant for sustained proliferation and motility of thyroid tumor cells by sequentially triggering RAS/BRAF/ERK activation (122). Enhanced activation of another signal-transduction route, the PI3K/AKT pathway, has also been reported in PTC (123). RET can activate AKT (via Y1062) through both PI3K-dependent and PI3K-independent mechanisms (68, 124). It is noteworthy that AKT activation is a common feature of aggressive thyroid cancers (125). The docking sites Y1015 and Y1062 are also required for stimulation of an osteopontin-CD44 autocrine loop initiated by RET/PTC. This loop activates ERK and AKT signaling pathways, is implicated in sustaining proliferation and invasiveness of thyroid cancer cells (126), and correlates with aggressive clinicopathological features of PTC (127).

RET/PTC signaling through Y905, Y1015, and Y1062 generally occurs independently of the type of rearrangement. However, there are some indications that different signaling cascades activated by the various RET/PTC rearrangements affect the clinical behavior of PTC. Miyagi *et al.* (128) have demonstrated that *RET/PTC3* expression (associated with more aggressive PTC) preferentially activates the PI3K/AKT rather than the RAS/BRAF/ERK pathway. Nevertheless, it is still unclear how these cascades lead to cellular changes seen in PTC.

The variable clinical behavior of *RET*-associated PTC may also be explained by a difference in expression levels of *RET/PTC* in aggressive and indolent tumors. In a report of a small series of PTCs, it was suggested that tumor size correlates with *RET/PTC1* expression levels, but this was not significant. Remarkably, expression levels of *RET/PTC* did not correlate with the presence of lymph node metastases or tumor stage (129).

Finally, the involvement of different proteins fused to RET may play a role in tumor behavior. In the clinically more aggressive tumors that are associated with *RET/PTC3* rearrangements, the fusion gene is *RFG* (also called *ELE1*). *ELE1* is a coactivator of peroxisome proliferator-activated receptor-γ (PPARγ), which has tumor suppressor possessions (130). This observation has led to the hypothesis that, upon rearrangement with *RET*, *ELE1* is inactivated as coactivator of PPARγ. Hence, in tumors containing *RET/PTC3* rearrangements, a proto-oncogene (*RET*) is activated and a tumor suppressor (PPARγ) could be inactivated (131).

Despite all efforts, thus far there is still little, if any, evidence whether and how the clinical behavior of human PTC is affected by the various RET/PTC rearrangements leading to activation of different downstream signaling proteins, differences in *RET/PTC* expression levels, or the involvement of different fusion genes.

4. RET, MEN 2, and FMTC. The MEN 2 syndrome consists of two variants: MEN 2A and MEN 2B. MEN 2A is characterized by medullary thyroid carcinoma (MTC; originating from the calcitonin-secreting parafollicular C cells of the thyroid gland) or its precursor C cell hyperplasia (CCH), pheochromocytoma (a tumor of the adrenal chromaffin cells), and hyperparathyroidism. Rarely, MEN 2A can be associated with cutaneous lichen amyloidosis (a pruritic and pigmented papular lesion of the skin on the upper back) or HSCR. MEN 2B is characterized by MTC, pheochromocytoma, mucosal ganglioneuromatosis, thickened corneal nerves, and a distinct marfanoid habitus. FMTC is characterized by MTC or CCH alone (132) but can also be associated with HSCR.

In 1987, the genetic defect causing MEN 2A was located on chromosome 10 (133). In 1993, it was demonstrated that MEN 2A and FMTC were caused by germline *RET* mutations (134, 135). Subsequently, it became clear that MEN 2B was caused by germline mutations in the *RET* proto-oncogene as well, whereas somatic *RET* mutations were detected in tumor tissue of approximately 40% of sporadic (nonfamilial) MTCs (70, 136–138).

The pattern of inheritance in MEN 2 and FMTC is autosomal dominant, and all patients carry germline point mutations in the *RET* gene. The clinical expression of the MEN 2 variants and FMTC varies (Table 1), but MTC is generally the first neoplastic manifestation because of its earlier and higher penetrance compared with pheochromocytoma or parathyroid hyperplasia (139, 140). This indicates that C cells are more susceptible to (oncogenic) RET activation than adrenal medullary or parathyroid cells. The disease phenotype

TABLE 1. Clinical expression of the variants of hereditary MTC-associated syndromes

	FMTC	MEN 2A	MEN 2B
MTC	100	100	100
CCH	100	100	100
Pheochromocytoma	0	10 to 60	50
Hyperparathyroidism	0	10 to 30	0
Cutaneous lichen amyloidosis	0	<10	0
HSCR	0	Rare	0
Marfanoid habitus	0	0	100
Intestinal ganglioneuromatosis	0	0	60 to 90
Mucosal neuromas	0	0	70 to 100
Thick corneal nerves	0	Rare	60 to 90
Age at presentation (yr)	<20 to >50	<20	<10

Data are expressed as percent unless otherwise specified.

correlates strongly with mutations in specific codons of *RET* (Fig. 5) (139–141) independent of the amino acid type substitutes (142, 143). MEN 2B is usually caused by mutations in the tyrosine kinase 2 subdomain (in 95% of cases involving codon 918 and in 5% codon 883). Infrequent germline missense mutations were reported at codons 804 and 806 in the same allele and also at codons 804 and 904 in the same allele, although the phenotype corresponding with the codon 804/904 double mutation does not meet the diagnostic criteria for MEN 2B (144, 145). MEN 2A and FMTC mutations affect primarily the extracellular cysteine-rich domain and are less frequently associated with mutations in the kinase domain (Fig. 5) (139, 146). In MEN 2A, codon 634 is most frequently affected (85%), mostly by a C634R substitution (which has never been found in FMTC), whereas in FMTC the mutations are more evenly distributed among the various codons (135, 140, 146). In 10 to 15% of MEN 2A and FMTC cases, codons 609, 611, 618, or 620 are affected, whereas in about 5% mutations do not reside in codon 609, 611, 618, 620, or 634. In these cases, patients carry rare mutations at the extracellular codons 321, 533, 600, 603, 606, 630, 649, and 666 (146–152) or the intracellular codons 768, 777, 778, 781, 790, 791, 804, 852, 891, and 912 (Fig. 5) (153–163). Some mutations (R321G, G533C, R600Q, K603E, Y606C, S649L, N777S, V778I, Q781R, I852M, and R912P) have only been associated with (F)MTC in a single pedigree (147–152, 154, 155, 160, 163). In addition, double *RET* mutations (C618S with E623K, C634Y with D631E, C634W with R635G, C634R with R640G, C634S with

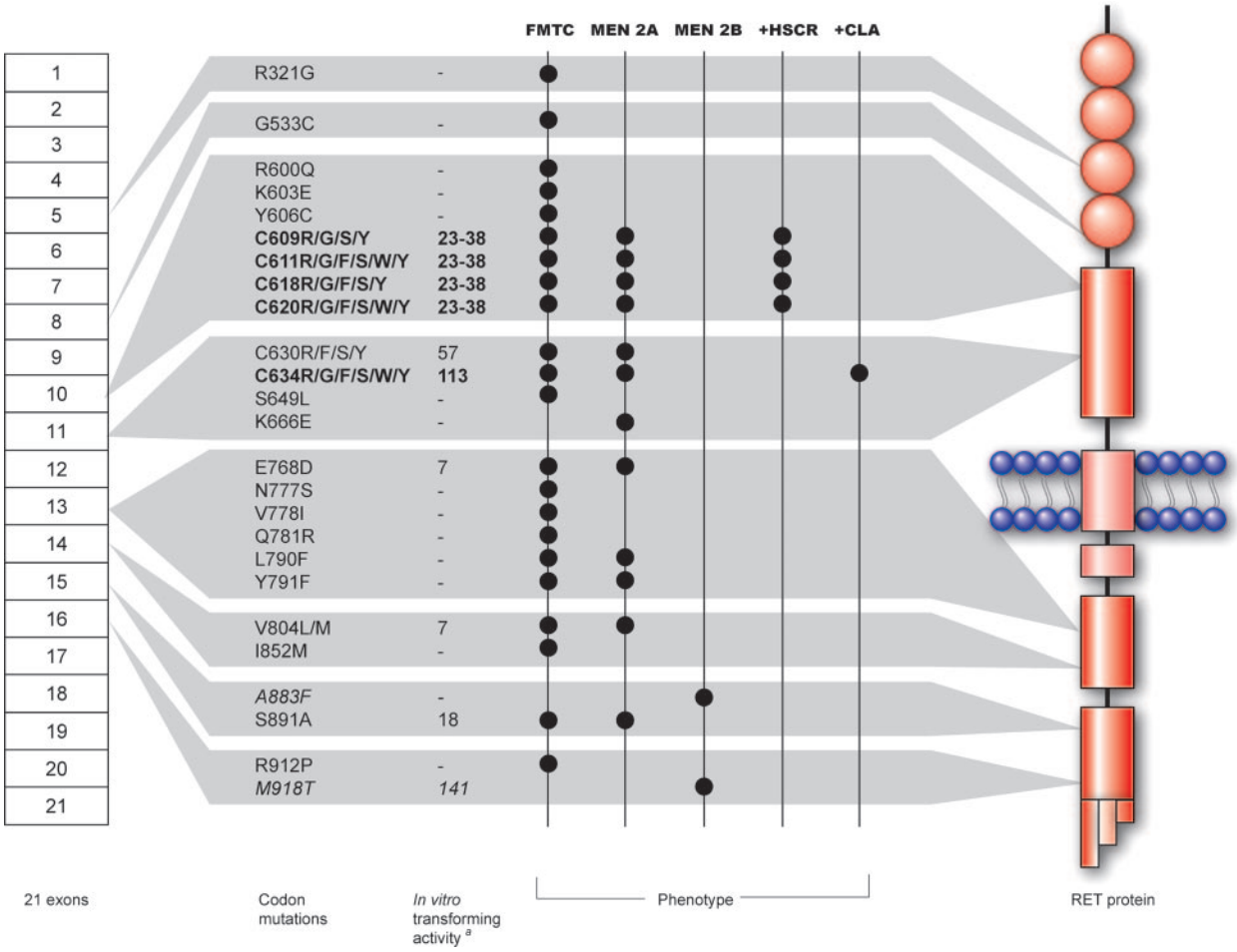

FIG. 5. Overview of the known germline missense mutations in the *RET* gene and their associated human diseases. The structure of the *RET* mRNA and the RET protein are depicted schematically. The mutations responsible for the diverse inherited cancer syndromes and the location of the mutations relative to the exons and the functional domains are shown. The most common mutations that are found in about 95% of MEN 2A and FMTC cases are depicted in *bold*, and MEN 2B mutations are depicted in *italics*. CLA, Cutaneous lichen amyloidosis. [[a] Derived from Ito *et al.* (142) and Iwashita *et al.* (74).]

A641S, C634R with V648I, and V804M with R844L), small insertions (in codons 532, 635, and 637), deletions (codon 616), and small insertion-deletion mutations (codons 631, 633, 635/636, 666, and 882) have been described in MEN 2A and FMTC (144, 152, 164–175). Experience in penetrance and aggressiveness is limited to a handful of pedigrees carrying these rare mutations, and genotype-phenotype correlations should therefore be interpreted with caution.

Some germline *RET* mutations are associated with MTC (or other endocrine tumors) only when they are present in a homozygous state, suggesting that these mutations have weak transforming capacities (176, 177). For example, mutations in codon 804 have variable clinical impact and can cause low penetrance disease, with late onset and a relatively indolent course (178) or more aggressive disease (179, 180). Individuals heterozygous for such weakly transforming mutations of *RET* likely require a second germline or somatic mutation in *RET*, a downstream signaling gene, or a tumor suppressor gene to result in clinical expression of MEN 2 (181). The occurrence of these second mutations and their transforming ability could account for the observed clinical variability in expression of germline mutations with very low transforming activity.

5. RET, MEN 2, and FMTC associated with HSCR. HSCR or colonic aganglionosis is characterized by the absence of the enteric ganglia along variable lengths of colon and is the main cause for congenital constipation with an incidence of 1 per 5000 live births. HSCR is a heterogenic disorder, because a number of genes have been shown to play a role in the disease etiology. To date, 10 genes have been associated with HSCR (182, 183). The major susceptibility gene is RET, in which mutations have been identified in 50% of familial and 15 to 35% of sporadic HSCR cases (182, 184). Most HSCR-associated mutations disable the activation or expression of RET (6), whereas the typical MEN 2 mutations result in constitutively active RET. Nevertheless, HSCR can be found in association with MEN 2A and FMTC in patients with a single point mutation at codon 609, 611, 618, or 620 (185).

6. RET and sporadic MTC. In 40 to 50% of sporadic MTCs, somatic RET mutations have been found. The most common mutation is M918T, although mutations at codons 609, 611, 618, 620, 630, 631, 632, 634, 636, 639, 641, 748, 766, 768, 876, 883, 884, 901, 908, 919, 922, and 930 and deletions including codons 592 to 607, 630, 632/633, 633 to 635, and 634 have also been described (135, 136, 138, 186–196). Sporadic MTCs show attributes of both MEN 2A and MEN 2B-related MTC (72). The contribution to tumor development of somatic RET mutations in MTC pathogenesis is unclear, although tumors with a somatic codon 918 mutation appear to be more aggressive (186, 189, 197). Somatic RET mutations are not consistently distributed within primary tumors and metastases, indicating that the mutation can occur during progression of the tumor or that MTC is a disease of polyclonal origin (138). Probably in these cases, somatic RET mutations merely contribute to the disease phenotype instead of causing it.

7. RET and sporadic pheochromocytoma. In apparent sporadic pheochromocytomas, the frequency of germline *RET* mutations ranges from 0 to 5% (198, 199). Somatic RET mutations have been found in 0 to 31% of tumors, mostly at codon 918 (136, 200–203) and appear to occur less frequently in malignant than in benign pheochromocytoma (200, 203). The contribution of germline and somatic *RET* mutations in the evolution of apparent sporadic benign and malignant pheochromocytomas therefore seems to be minimal, and other genes likely play a more important role in tumorigenesis of pheochromocytoma.

8. Oncogenic RET activation in sporadic and hereditary neuroendocrine tumors. Mutated RET plays a very significant role in the development of human neuroendocrine tumors and tumor syndromes. Oncogenic RET activation and signaling differs from activation and signaling of nonmutated RET. These differences in the various neuroendocrine tumors will be described next.

In MEN 2 and FMTC, the activation of oncogenic RET depends on the location of the amino acid change. Mutations in the extracellular cysteine-rich domain are generally found in MEN 2A (Fig. 5) and convert a cysteine residue into a noncysteine residue. Normally, these cysteine residues are involved in intramolecular disulfide bonds in wild-type RET. The mutation leaves an unpaired cysteine residue in a RET monomer to form an aberrant intermolecular disulfide bond with another mutated monomer. The two mutated RET molecules are constitutively dimerized and activated in *trans*. Mutations in the intracellular tyrosine kinase domain, which are generally found in MEN 2B and FMTC (Fig. 5), activate tyrosines in the kinase domain and alter its substrate specificity due to structural changes of the binding pocket of the tyrosine kinase domain. They lead to aberrant phosphorylation of substrates preferred by cytoplasmic tyrosine kinases such as c-Src and c-abl rather than the substrates preferred by normal receptor tyrosine kinases (36, 204). Consequently, the mutated RET no longer needs dimerization to become active (205).

It is remarkable that, although mutated RET signals independent of ligand, in several mutation types RET can be further activated by GDNF (206). MEN 2B-associated intracellular mutations, for instance, could be activated by GDNF as opposed to intracellular FMTC mutations. This same phenomenon was observed for extracellular codon 634 mutations that were responsive to GDNF, whereas codon 620 mutations were not (207).

Little is known about the (mutation-specific) signaling pathways of RET. There may be subtle differences in protein conformation when RET is activated by ligand binding, MEN 2A mutations, MEN 2B mutations, or FMTC mutations leading to the initiation of different intracellular signaling pathways. Wild-type RET, MEN 2A-related RET (RET/MEN 2A), FMTC-related RET (RET/FMTC), and MEN 2B-related RET (RET/MEN 2B) display differences in phosphorylation of docking sites and isoforms of the RET receptor (71, 208, 209). In RET/MEN 2, a variable pattern of phosphorylation, including docking sites Y752, Y905, Y928, and Y1096 has been identified (35, 208). Phosphorylation of Y752 and Y928 results in activation of STAT3 in RET/MEN 2A and RET/FMTC (35, 36), and the transforming activity of RET/MEN 2A but not RET/MEN 2B depends on phosphorylation of Y905 (37). With regard to Y1096, it has been demonstrated that in RET/

MEN 2B, Y1062 phosphorylation is enhanced and Y1096 phosphorylation is reduced, whereas in RET/MEN 2A, Y1096 phosphorylation is enhanced (30).

These differences in phosphorylation of docking sites and response to GFLs may give rise to altered activation of downstream signaling routes. This seems indeed to be the case. RET/MEN 2A, for instance, impacts substantially on downstream AKT activation compared with RET activated by its natural ligand (210). Several additional findings suggest that different mutated RET proteins might have different effects on tumorigenesis. The PI3K/AKT pathway responsible for survival signaling, enhanced cell-cycle progression, and RET-mediated transformation is more highly activated in RET/MEN 2B than in RET/MEN 2A (48, 49). Because of the enhanced Y1062 phosphorylation of RET/MEN 2B compared with RET/MEN 2A, higher activation levels of the RAS/MAPK and PI3K/AKT pathway are triggered (211). These observations suggest that PI3K/AKT is (one of the) most important oncogenic signaling pathways.

Further evidence for differences in oncogenic signaling between the various mutation types is provided by the strong association of the JNK pathway with RET/MEN 2B and involvement of this pathway in the ability of MEN 2B-related MTC to metastasize (62, 63). Moreover, the activation of STAT3 by an extracellular RET/MEN 2A mutation is independent of Janus tyrosine kinases and c-Src. In contrast, RET^{Y791F} and RET^{S891A} (intracellular monomeric FMTC/MEN 2A mutations) activate STAT3 via c-Src and Janus tyrosine kinases (36).

The behavior of MEN 2 and FMTC-related MTC subtypes can be coupled to specific gene expression profiles. Screening analysis using an *in vitro* model of NIH3T3 cells expressing RET/MEN 2A and RET/MEN 2B identified 10 genes that were induced by both mutations, and eight genes were repressed (59). The induced genes included cyclin D1, cofilin, and cathepsin L and B, which are known to be implicated in cell growth, tumor progression, and invasion. The repressed genes included type 1 collagen, lysyl oxidase, annexin 1, and TIMP3 genes that have been associated with tumor suppression. Furthermore, RET/MEN 2A predominantly induced six genes, and RET/MEN 2B predominantly induced five genes. Among these genes, ITGA6 expression has been suggested to play a role in the MEN 2A phenotype, and STC1 in the MEN 2B phenotype. Expression microarray analysis of human MEN 2A- and MEN 2B-related MTC demonstrated up-regulation of a cluster of genes associated with matrix remodeling and the epithelial to mesenchymal transition. These and other gene products in the MEN 2B cluster have been previously associated with an increased metastatic potential in a variety of other tumors, including breast, prostate, and bladder carcinomas (72).

9. Oncogenic RET activation in MEN 2 and FMTC associated with HSCR. MEN 2A and FMTC can cosegregate with HSCR, and these phenotypes are, in these cases, caused by the same *RET* mutation. Several observations have been made that could offer an explanation for this apparent contradicting phenomenon, including a decreased cell surface expression of RET in these patients (212) and a kinase activity under a certain threshold required for cell survival (142). However, the impact of GDNF-mediated signaling may influence oncogenic signaling. As described above, pure MEN 2A mutations such as C634R are responsive to GDNF, whereas HSCR/MEN 2A- and HSCR/FMTC-mutated RET (for instance C620R) does not respond to GDNF (207). Insensitivity to GDNF renders cells more prone to apoptosis, and these features are shared by all HSCR-associated mutations of *RET* (213). Unlike the HSCR/MEN 2A mutations, pure MEN 2A mutations such as the C634R mutation are responsive to GDNF and are therefore most likely not associated with HSCR (207). A similar influence of GDNF has been demonstrated for MEN 2B-associated RET^{M918T} as opposed to FMTC-associated RET^{Y791F} and RET^{S891A}. RET^{M918T} displays larger oncogenic potential and has been shown to be GDNF-responsive, whereas RET^{Y791F} and RET^{S891A} are not (36). These findings suggest that differences in the mechanism of receptor activation combined with differences in GDNF responsiveness of these receptors, as well as tissue-specific expression of GDNF (or related ligands), could give rise to different disease phenotypes (214).

VI. *RET* Polymorphisms and Haplotypes in Endocrine Tumors

Common polymorphic variants of RET can also contribute to the disease phenotype. A genetic locus is considered polymorphic if one or more of the rare alleles has (have) a frequency of at least 0.01. Most polymorphisms do not alter the functional activity of the encoded protein, but not all polymorphisms are neutral. If the presence of a polymorphism or haplotype (a set of closely linked markers or polymorphisms inherited as a unit) correlates (or associates) with a certain phenotype, it might be that it acts as a genetic modifier and may be associated with a (small to moderate) increased relative risk for the development of the disease. It might also be that polymorphisms interact with other genetic variants and with traditional germline MEN 2-associated mutations to modulate development of features and age at onset. Moreover, because polymorphisms are relatively common in the population, they may present a much higher attributable risk in the general population than rare mutations in high penetrance cancer susceptibility genes such as *RET*.

A. Papillary thyroid carcinoma

Only a few studies of an association between *RET* polymorphisms and haplotypes and PTC have been reported so far (215–217). These studies demonstrated a weak association with PTC and the single nucleotide polymorphisms (SNPs) A45A, L769L (215), A432A (217), G691S, and S904S (216). Furthermore, analysis of haplotype frequencies suggested that one specific haplotype, named the GGCC haplotype, may act as a low penetrance predisposing allele for PTC in the Italian and French populations (215). However, all things considered, the magnitude of the effect between the *RET* SNPs/haplotypes and PTC is quite modest at best and should be confirmed on larger samples.

B. Multiple endocrine neoplasia type 2 and familial medullary thyroid carcinoma

Because both related and unrelated individuals with the same germline *RET* mutations develop MTC (and pheochromocytoma) at different ages, other genetic or epigenetic events may trigger tumorigenesis, including the presence of *RET* polymorphisms and ancestral haplotypes. Several SNPs and haplotypes of *RET* have been described in the general population (218) and in association with MEN 2A (219). A recent study suggested that the polymorphic G691S/S904S variant of *RET* has a modifier effect on the age at which MEN 2A begins (219), and another recent study suggested an association of the SNP L769L with the FMTC germline mutation F791Y (220). Nevertheless, the mechanism of action of these potential genetic modifiers remains to be demonstrated.

C. Sporadic medullary thyroid carcinoma

Several *RET* polymorphisms have been described in sporadic MTC. In a study among sporadic cases of MTC from Germany and the United States, the SNP S836S was overrepresented and apparently associated with the somatic mutation M918T in the tumoral DNA from the same patients (221). These results were independently confirmed in another study of Spanish MTC patients (222). However, in other studies of French, Polish, British, Chilean, and Austrian patients, respectively (220, 223–226), the S836S polymorphism was not found associated with predisposition to sporadic MTC.

The IVS1–126G→T polymorphism was significantly overrepresented in Spanish patients with sporadic MTC, and the disease is associated with a specific haplotype within *RET* intron 1 that contains IVS1–126G→T and IVS1–1463T→C (227). However, the association between this SNP and sporadic MTC was excluded in UK patients (225).

The association of haplotype CGGATGCCAA and sporadic MTC was recently demonstrated in patients from the United Kingdom. This haplotype harbors the SNPs G691S, S904S, and STOP + 388 bp on exon 19 (225). G691S and S904S have previously been associated with sporadic MTC and MEN 2A (176, 219) and G691S is thought to be the functional polymorphism. It was hypothesized that the G→S amino acid change creates a new phosphorylation site, which affects downstream signaling (219). It could also be that the SNP changes the secondary structure of RET, affecting flexibility and solvent accessibility of the protein (225). Further experimental data, however, are needed to verify these hypotheses. It is of note that the germline sequence variant in intron 14 (IVS14–24G→A), originally interpreted as a disease causing mutation for HSCR (228), has also been found in a significantly higher frequency in patients with sporadic MTC and in subjects with moderately elevated serum calcitonin concentrations after pentagastrin stimulation, when related to a control group (220). In contrast, IVS14–24G→A was not associated with either HSCR or sporadic MTC in another study (229). Interestingly, a haplotype with a protective effect for sporadic MTC was recently identified (225). This haplotype contained the SNP A45A, which was previously associated with an increased risk of HSCR (230). Despite these findings, it is unlikely that A45A is responsible for this protective effect because it was also present in a haplotype that lacked association with sporadic MTC (225).

Furthermore, GFRα1–193, a polymorphism of the *GFRα1* gene, was found to be associated with sporadic MTC in a small case-control study (231). However, in two larger studies, this association could not be reproduced (225, 232).

The potential role of the different polymorphisms in the development of sporadic MTC needs to be further characterized, and the molecular background of these polymorphisms needs to be elucidated.

D. Sporadic pheochromocytoma

An ancestral, low-penetrance *RET* haplotype is strongly associated with and overrepresented in sporadic pheochromocytoma. It comprises the wild-type allele at IVS1–126 and IVS1–1463, with a 16-bp intron 1 deletion 5′ of these SNPs (233). In addition, a significant association between the patients' age at diagnosis and genotype was found, suggesting that the additive effect of the haplotypes can modulate the age of onset of the disease.

VII. Diagnostic and Therapeutic Implications of the *RET* Genotype in Multiple Endocrine Neoplasia Type 2 and Familial Medullary Thyroid Carcinoma

Patients who present with MTC should undergo genetic screening for germline *RET* mutations because the likelihood of a familial component is relatively high (2.5 to 7% in apparent sporadic MTC; Ref. 146) and early thyroidectomy proved to be the only effective curative or preventive treatment (234–236). In the assessment of at-risk individuals, DNA analysis for the detection of mutations in the *RET* gene is the gold standard, and a positive result is the single indication for recommending surgery (172). Genetic screening includes the analysis of exons 10, 11, 13, 14, 15, and 16 because the clinically relevant mutations are located in these exons (140). The recent discovery of a *RET* germline mutation in codon 321 in exon 5 (147) and codon 533 in exon 8 (148) indicates that analysis of exons 5 and 8 should be considered in patients and families at risk for MEN 2 and without identified mutations in exons 10, 11, 13, 14, 15, and 16 and maybe even in every patient presenting with MTC.

MTC has nearly a 100% penetrance in MEN 2 syndromes and FMTC, but the aggressiveness and clinical course differ between the different types of MEN 2. Therefore, based on recent literature, *RET* mutations have been stratified into three groups, levels 1 to 3. Patients with MEN 2B have the most aggressive MTCs (mutations in codon 883 or 918). They are classified as level 3. Patients with MEN 2A/FMTC-related level 2 mutations (codon 609, 611, 618, 620, 630, 634) are at high risk, and patients with *RET* codon 768, 790, 791, 804, and 891 (level 1) mutations are classified as having the least high risk for the development and growth of aggressive MTC (140, 143). The biological behavior of MTC observed in patients with level 1 mutations, however, is variable, and MTC with lymph node metastases has been reported even at the age of 6 in these patients (143). Recently, new insights re-

garding average tumor behavior in MEN 2/FMTC kindreds with a particular mutation regarding the development of MTC and pheochromocytoma have been described (141, 143, 237–239). Timing of screening and treatment for MEN 2-associated tumors may now be based on the type of *RET* mutation in patients with a MEN 2/FMTC genotype. A treatment and screening strategy based on the earliest occurrence of MTC, pheochromocytoma, and primary hyperparathyroidism for carriers of germline *RET* mutations as well as the *in vitro* transforming capacities of the different mutations (74, 142) (Fig. 5) is depicted in Table 2. Total thyroidectomy and central lymph node dissection should be performed in the first year of life in patients with level 3 mutations because MTC is present very early and these patients have a high risk of lymph node metastases (240). In asymptomatic carriers of level 2 mutations, total thyroidectomy is generally recommended before the age of 5, although based on the youngest age of occurrence of MTC and the *in vitro* transforming activity of the mutations, surgery is warranted before the age of 2 in patients with a mutation in codon 630 or 634. It should be noted, however, that all reported patients with a codon 630 or 634 mutation who have been operated around the age of 4 or 5 yr had undetectable serum calcitonin levels postoperatively (140, 143, 172, 234, 236, 241–248). Finally, in asymptomatic carriers of level 3 mutations, total thyroidectomy is recommended before the age of 10.

There is still a great amount of controversy regarding the issue of when to perform a central lymph node dissection (140). The interval between the emergence of MTC and the evolution of lymph node metastases has been estimated to be 6.6 yr for carriers of the most common mutations in codon 634 (143). Lymph node metastases are uncommon before the age of 10 yr in patients harboring mutations in codon 630 or 634 (143, 235) and before the age of 20 yr in patients with mutations in codon 609, 611, 618, 620, and 912 and the level 1 mutations (143, 163, 235). However, individual predictions of phenotype can be very unreliable based solely on *RET* genotype. This is illustrated by the emergence of MTC with lymph node metastases in two patients, respectively 5 and 10 yr old, with a *RET* mutation in codon 634 (249, 250), a 6-yr-old patient with a codon 804 mutation (251), and a 10-yr-old patient with a codon 790 mutation (252). These patients all had abnormal basal and/or stimulated serum calcitonin levels. Therefore, it is advisable to perform a total thyroidectomy including a central compartment dissection, irrespective of the patient's age in case of abnormal calcitonin levels.

In MEN 2A patients with a codon 634 *RET* mutation, pheochromocytomas have been identified as early as 5 and 10 yr of age (140). However, the recent results of a single institute cohort study suggest a later age of onset and a codon-specific, age-related development of MEN 2-associated pheochromocytoma (237). Based on a worst case scenario, screening for pheochromocytoma through the annual measurement of urinary catecholamines and metabolites should commence before the earliest reported age of presentation. Therefore, except for patients with a *RET* mutation in codon 634 (and presumably also in codon 630), who should be screened from the age of 5 yr onward, screening for pheochromocytoma may be postponed until the age of 20 in patients with level 1 and 2 mutations. Likewise, screening for primary hyperparathyroidism (serum calcium and PTH) should commence before the age of 10 in carriers of a codon 634 and codon 804 mutation and could be postponed to the age of 20 yr in other mutation carriers. For pragmatic reasons, screening for pheochromocytoma and primary hyperparathyroidism could best be combined.

TABLE 2. Management of MEN 2 and FMTC patients according to *RET* genotype[a]

Risk category	Risk level	*RET* codon	Youngest age at first diagnosis			Recommended age for surgery (yr)		Recommended age to start screening (yr)	
			MTC	PCC (yr)	HPT (yr)	Thyroidectomy	Central lymph node dissection	PCC	HPT
Highest	3	883	Not described	Not described		<1	<1	10	
	3	918	9 months	12		<1	<1	10	
High	2	609	5 yr	22	Unspecified	<5	≥20[b]	20	20
	2	611	7 yr	30	Unspecified	<5	≥20[b]	20	20
	2	618	7 yr	29	41	<5	≥20[b]	20	20
	2	620	6 yr	22	Unspecified	<5	≥20[b]	20	20
	2	630	12 months		32	<2	≥10[b]	5	20
	2	634	13 months	5	10	<2	≥10[b]	5	<10
	2	912	14 yr			<5	≥10[b]	20	20
Least high	1	533	21 yr			5–10	≥20[b]	20	20
	1	649	44 yr			5–10	≥20[b]	20	20
	1	666	35 yr	35		5–10	≥20[b]	20	20
	1	768	22 yr	59		5–10	≥20[b]	20	20
	1	790	10 yr	28		5–10	≥20[b]	20	20
	1	791	21 yr	38	38	5–10	≥20[b]	20	20
	1	804	6 yr	28	10	5–10	≥20[b]	20	<10
	1	891	13 yr	52		5–10	≥20[b]	20	20

For mutations at codons 321, 603, 606, 777, 778, 781, and 852, insufficient data are available for recommendations, but most likely they belong to risk level 1. PCC, Pheochromocytoma; HPT, hyperparathyroidism.

[a] The recommendations are based on the 1999 International Consensus Statement (140) and extended with results from recent literature (141, 143, 151, 152, 163, 180, 235, 237).

[b] No consensus has been reached for the extent of surgery for MTC in patients carrying these germline mutations in the *RET* gene. Recommendations are based on recent literature. If basal or pentagastrin-stimulated calcitonin levels are abnormal in *RET* mutation carriers, thyroidectomy and central lymph node dissection should be performed immediately.

VIII. RET as a Therapeutic Target

A. Current treatment options for RET-associated endocrine tumors

The current recommended treatment for PTC is total thyroidectomy followed by adjuvant ^{131}I therapy. Alternative treatment options have limited effect (76). In general, this treatment strategy is safe. However, in around 20% of patients, treatment is unsuccessful, and patients with persistent disease have a median life expectancy compared with the general population of only 60% (253). In MTC and pheochromocytoma, surgically removing all neoplastic tissue is the only treatment option with curative intent. Once MTC and, in rare cases, MEN 2-associated malignant pheochromocytoma has metastasized, there are no therapeutic options (254) although ^{131}I-meta-iodobenzylguanidine therapy can provide long-term palliation in disseminated malignant pheochromocytoma (255). Therefore, there is an urgent need to search for new kinds of treatment.

B. Various ways to inhibit RET signaling

Although the impact of inhibition of RET signaling on normal untransformed cells is poorly understood, it seems an attractive option, especially because the adverse effects of several potential RET inhibitors that have been evaluated in clinical trials appear to be limited and manageable. In preclinical literature, several ways to block different steps in the functioning of tyrosine kinases have been developed (Fig. 6). In the above, crucial steps in the activation and signaling of RET have been described, including the formation of ligand-coreceptor (GFR-GFL) complexes, dimerization, autophosphorylation of RET, recruitment of adaptor proteins to various docking sites, and initiation of signal transduction cascades. Down-regulation leading to the disappearance of RET from the cell surface constitutes another important means of regulation and a potential target for therapy. All these steps may be subject to specific inhibitors. Furthermore, several therapeutic options regarding the biosynthesis of RET have been described (256). Still, many ways of inhibition have not been tested on RET or clearly exploited in drug candidates so far. Next, we will present and discuss an overview of current developments in therapeutic drugs aiming at attenuating RET signaling.

C. Ligand binding: antagonists and monoclonal antibodies

The first step in the activation of RET is binding of GFL to the GFR coreceptor. Targeting GFLs or the binding site of the coreceptor therefore represents a straightforward approach for RET inhibition. Two synthetic agents (GFB-111 and GFA-116) that selectively bind to platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), respectively, disrupt binding to their receptor-tyrosine kinase [PDGF receptor (PDGFR) and VEGF receptor (VEGFR)] and inhibit the oncogenic signaling function of both growth factors *in vitro* and *in vivo* (257, 258). However, of several (co-) receptor antagonists that have been developed, none targets RET or its coreceptors.

Monoclonal antibodies against the GFLs seem an attractive target as well, because MEN 2B-associated RET^{M918T} and MEN 2A-associated RET^{C634R} are still responsive to GDNF. However, such antibodies do not yet exist for RET.

D. Dimerization: inhibitors

The second step in RET activation is dimerization. A few studies have shown that small peptides can be used to inhibit dimerization of receptor tyrosine kinases that are implicated, via a mutation or overexpression, in human cancer (259, 260). It is thought that the transmembrane domains of receptor tyrosine kinases are directly involved in receptor dimerization and activation. If this is indeed the case, then introduction in the membrane of peptides corresponding to the transmembrane domain should compete with dimerization and thus inhibit the kinase activity. In human cancer cells overexpressing ErbB2 (also called HER2 or Neu) and epidermal growth factor receptor tyrosine kinases, expression of small transmembrane peptides indeed inhibited the tyrosine kinase activity (261). Whether similar inhibitory approaches also may be applicable to other receptor tyrosine kinases remains to be studied.

With the systematic evolution of ligands by exponential enrichment technology, specific oligonucleotide ligands (aptamers) can be generated. These aptamers can be used to identify markers on the surface of a cell type, define the specificity of a cellular state, and allow *in vivo* targeting for diagnostic and therapeutic applications (262). Aptamers are specific, have a high affinity for their target, are poorly immunogenic, and can recognize a wide variety of targets. The neutralizing, nuclease-resistant D4 aptamer was capable of binding and inhibiting wild-type RET and RET/MEN 2A on the cell surface. The fact that the monomeric RET/MEN 2B was not affected suggests that D4 acts by interfering with the formation of a stable, active RET dimer (263). Several compounds obtained by systematic evolution of ligands by exponential enrichment are currently under clinical trials (264). However, the efficacy of D4 as a therapy for RET-associated tumors needs to be established.

E. Autophosphorylation: tyrosine kinase inhibitors

Dimerization of the receptor due to ligand activation or mutations results in autophosphorylation of the tyrosine kinase domain. Although tyrosine kinases play a critical role in diverse physiological processes, recent successes in targeting a variety of tyrosine kinases in cancer (265) have drawn attention to RET as a possible therapeutic target. The role of RET in oncogenesis is emphasized by its unique function in the development and growth of neuroendocrine tumors and the fact that expression of oncogenic RET alone is enough to transform NIH3T3 fibroblasts; therefore, RET seems an obvious target for intervention (205, 266).

The emergence of imatinib (signal transduction inhibitor 571) as a prototype of designer tyrosine kinase inhibitors demonstrated that tyrosine kinase inhibition can be rather specific and effective. Imatinib belongs to the 2-phenylaminopyrimidine class and proved to target constitutive active breakpoint cluster region-Abelson murine leukemia viral oncogene homolog (BCR-ABL), PDGFR, and SCFR (stem cell

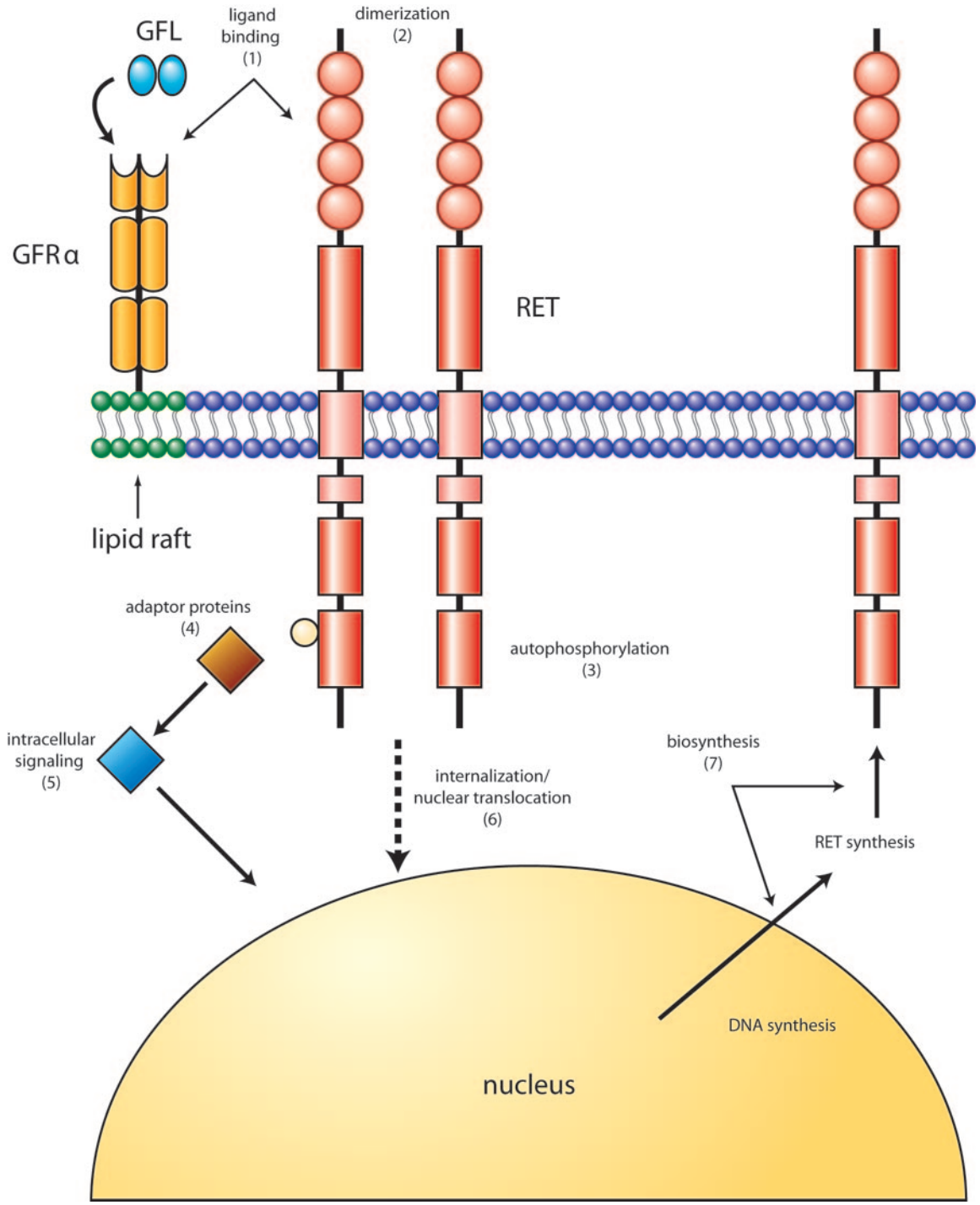

FIG. 6. Strategies to inhibit RET. The different steps involved in RET activation, regulation, and synthesis are schematically depicted. Each step is associated with a potential means of inhibition for therapy. 1, Ligand binding and formation of ligand-GFR complex (antagonists, antibodies); 2, receptor dimerization (inhibitors); 3, autophosphorylation (tyrosine kinase inhibitors); 4, recruitment of adaptor proteins (phosphatases, inhibitors of protein-protein interaction); 5, intracellular signaling (various inhibitors); 6, internalization and nuclear translocation (antibodies, inhibitors); and 7, biosynthesis (gene therapy, RNAi).

factor receptor, also called c-kit) tyrosine kinases. Currently, imatinib is clinically used to treat chronic myelogenous leukemia and gastrointestinal stromal tumors as well as dermatofibrosarcoma protuberans (265, 267, 268). Therefore, and because of similarities between RET and, for instance, c-kit, there is a rationale to select tyrosine kinase inhibitors for treatment of RET-mediated endocrine tumors.

Over the last several years, a couple of tyrosine kinase inhibitors have been demonstrated to inhibit RET activity. Imatinib has been shown to display inhibitory activity

against RET/MEN 2A and RET/MEN 2B in MTC-derived cell lines. Furthermore, it induces RET oncoprotein degradation. However, the IC_{50} (the concentration that causes 50% growth inhibition) of imatinib necessary to inhibit RET *in vitro* is high (25–37 μM). Hence, it is impossible to conclude that imatinib will be a good candidate for systemic therapy of MTC (269–271).

The 2-indolinone derivative ribose-5-phosphate isomerase (RPI-1) inhibited RET in human TT cells (a MTC-derived cell line harboring a RET^{C634W} mutation) and RET/MEN 2A and RET/PTC1-expressing NIH3T3 cells with an IC_{50} in the low micromolar range. RPI-1 decreased activation of downstream molecules including PLCγ, ERK, and AKT and reduced proliferation. RPI-1 also showed antitumor effects in nude mice and could be administered orally (272–274). The closely related compound SU11248 (sunitinib) is also a highly active inhibitor of RET and is currently evaluated in clinical trials for gastrointestinal stromal tumors and renal cell carcinoma (124, 275).

The pyrazolopyrimidines PP1 and PP2 inhibited enzymatic activity and transforming ability of NIH3T3 fibroblasts transfected with almost all types of RET/MEN 2A and RET/MEN 2B as well as RET/PTC1 and RET/PTC3 with an IC_{50} in the nanomolar range (276–279). However, PP1 and PP2 also inhibit the Src family of kinases. Therefore, the actions of PP1 and PP2 on oncogenic activity may not depend solely on RET inhibition. PP1 induces RET/MEN 2A and RET/MEN 2B oncoprotein degradation via proteasomal targeting (278), whereas imatinib induces RET degradation through nonproteasomal pathways (271). In addition, the pyrrolopyrimidine AEE788, which is a potent inhibitor (IC_{50} in the low nanomolar range) of VEGFR and epidermal growth factor receptor, has a RET IC_{50} of 740 nM (280).

The indolocarbazole derivatives CEP-701 and CEP-751 inhibited RET autophosphorylation and proliferation of TT cells at concentrations lower than 100 nM. Moreover CEP-751 inhibited tumor growth in nude mice that have been injected with TT cells (281).

The selective inhibitor of the VEGFR-2 tyrosine kinase ZD6474 is a member of the anilinoquinazoline family. ZD6474 targets the enzymatic activity of both MEN 2 and PTC-related oncogenic RET and has an IC_{50} of 100 nM. In addition, the compound inhibits tumor growth in RET/PTC-transformed NIH3T3 cell xenografts (282). Because ZD6474 inhibits the VEGFR-2, it has antiangiogenic capacities as well. Moreover, it has proven to have few side effects despite the lack of selectivity for a certain kinase (283). The preliminary results of an ongoing phase II study with ZD6474 in hereditary MTC are encouraging (284), and further data will be awaited.

Due to their powerful inhibitory effects, RPI-1, SU11248, PP1, PP2, AEE768, ZD6474, CEP-701, and CEP-751 seem a promising treatment strategy in RET-associated cancer. Other small molecule tyrosine kinase inhibitors with affinity for RET are expected to emerge in the near future.

Although most of the tyrosine kinase inhibitors examined lack selectivity for RET (or any other kinase), adverse effects are generally acceptably low and manageable. Therefore, tyrosine kinase inhibitors might soon become the therapy of choice for RET-associated thyroid cancer. However, resistance to tyrosine kinase inhibitors may occur due to mutation and amplification of the target kinase, which leads to interference with binding of the inhibitor. In RET, replacing valine at codon 804 with the amino acids leucine or methionine indeed mediated resistance to pyrazolopyrimidines and anilinoquinazolines (279). Combining different (kinase) inhibitors may overcome this resistance.

F. Recruitment of adaptor proteins: phosphatases

Activation of phosphatases leading to accelerated removal of substrate phosphates has been demonstrated to counter kinase signaling (285). It has been shown that Src homology 2-containing protein tyrosine phosphatase-1, a cytoplasmic protein phosphotyrosine phosphatase, can associate with mutated RET, reducing its autophosphorylation rate, with consequent suppression of the growth-promoting signals by the RET-induced MAPK pathway (286, 287). This reduction in autophosphorylation is activated by somatotropin release-inhibiting factor (287). In light of these results, Src homology 2-containing protein tyrosine phosphatase-1 could represent a molecular target for future treatment of RET-associated cancer.

G. Intracellular signaling: inhibitors

Activated tyrosine kinases induce numerous downstream events and initiate different signaling pathways (Fig. 3). Specific key proteins (often kinases) involved in these pathways can be inhibited, resulting in modulation of the effects caused by the (mutant) tyrosine kinase. Several inhibitors can target the RAS/Ras effectors serine/threonine kinase (RAF)/MEK/ERK pathway for instance, which is involved in cell proliferation and differentiation. Several of these agents are under clinical evaluation in tyrosine kinase-associated human cancer (for review, see Refs. 288 and 289), although none of these agents has yet been investigated for RET. Of particular interest in RET-associated tumors is the biaryl urea BAY 43–9006 (sorafenib). BAY 43–9006 is an oral multikinase inhibitor initially developed as a specific inhibitor of BRAF. However, subsequent studies revealed that BAY 43–9006 also inhibits other kinases including VEGFR-2, VEGFR-3, PDGFR-β, c-kit, and FMS-like tyrosine kinase 3 (FLT-3). In addition, the compound also inhibits RET (290). Recently, it was demonstrated that BRAF is a key mediator of the intracellular signaling of oncogenic RET in follicular thyroid cells (122). The simultaneous action at two levels of the same signaling pathway (RET and BRAF) in endocrine tumors may offer perspective and a potential mechanism to circumvent the development of treatment resistance.

Other agents that interfere with the RAS/RAF/MEK/ERK pathway can be functionally characterized as inhibitors of *RAS* expression (antisense oligodeoxynucleotides), RAS processing (prenyltransferase inhibitors), and inhibitors of downstream effectors of RAF and MEK (small molecule inhibitors).

RAS [or any other gene, such as *RAF* and *PKC* (288)] expression can be altered at the transcriptional stage by use of oligonucleotides that cause the formation of triple helixes. An alternative strategy is to use single-stranded oligonucle-

otides, or antisense oligonucleotides, to modify gene expression at the translational step. LY900003 (Affinitak) is an antisense oligonucleotide, known to modify *PKC* expression by interacting with the mRNA involved in the production of disease-specific proteins and is currently in advanced clinical development (288). However, antisense therapeutics have shortcomings in specificity and consistency (291).

Many prenylated proteins are involved in signal transduction circuits whose dysfunction leads to cancer, including RAS. RAS proteins require posttranslational modification by prenylation to be biologically active. Prenyltransferase inhibitors have some antitumor activity in the clinic, but the antitumor activity cannot be ascribed simply to inhibition of RAS (292). Therefore, the crucial targets of prenyltransferase inhibitors remain to be identified.

Inhibitors of other downstream signal transduction proteins thus far have not been tested in RET signaling. Nevertheless, they provide interesting possibilities for future research and therapeutic options in *RET*-associated endocrine malignancies. A potential objective for intervention is the PI3K/AKT pathway responsible for proliferation, differentiation, and cell survival. The involvement of this pathway in oncogenesis is reviewed by Vivanco and Sawyers (293). A downstream substrate of AKT is mTOR, which is involved in the translation of growth regulatory gene products (294). mTOR integrates input from multiple upstream pathways and inhibition of mTOR (via the immunosuppressive drug rapamycin and its derivatives) results in cell cycle arrest and growth inhibition in a variety of human tumor systems (295).

The PLCγ/PKC cascade is targeted by several kinase inhibitors, antisense oligonucleotides, and staurosporine analogs (288). Although these classes of inhibitors have minimal single-agent activity, they can be administered safely with conventional chemotherapeutical regimens, and they have an additional antitumor effect in various human cancers (289, 296).

Several compounds containing platinum (297) as well as natural products (298) block STAT3 activity *in vitro* and *in vivo* at low micromolar concentrations. In malignant cells that harbor constitutively activated STAT3, these compounds inhibit cell growth and induce apoptosis. By contrast, cells that do not contain persistent STAT3 activity are marginally affected or are not affected (297, 298). Whether these STAT3 inhibitors are useful in clinical practice is not yet established.

JNK can be inhibited by the selective JNK inhibitor SP600125. JNK inhibition seems to lead to cell cycle arrest and CD95 mediated apoptosis, but the concentrations of inhibitor needed to achieve this effect are fairly high (299).

H. Internalization and nuclear translocation: antibodies

In an analogy of the successes of the monoclonal antibody trastuzumab (Herceptin) in breast cancer (300), targeting RET with a monoclonal antibody seems to be useful for MTC and PTC patients. Yano *et al.* (301) have already generated an antibody that is capable of internalization of RET, but its efficacy remains to be shown.

I. Biosynthesis: gene therapeutic and antisense approaches

Fascinating new viewpoints for treatment of MTC have been opened by novel gene therapeutic approaches. Gene therapy involves the integration of new genetic material into the genome. This approach can be used to replace defective genes or block the effects of unwanted ones by the introduction of a counteracting gene.

Inhibition of oncogenic RET signaling by expression of a dominant-negative *RET* mutant is an example of corrective gene therapy and has been investigated by Drosten *et al.* (302, 303). Adenoviral vectors expressing dominant-negative *RET* mutants were used. Because of amino acid changes in the cadherin domains of these dominant-negative RET mutants, the glycosylation process is disturbed, resulting in interference with protein transport to the cell surface. In addition, the dominant-negative mutants dimerize with oncogenic RET in the endoplasmic reticulum, thereby preventing expression of both dominant-negative and oncogenic RET on the cell surface. Using an adenoviral vector expressing dominant-negative *RET* leads to strong inhibition of cell viability caused by induction of apoptosis *in vitro*. Moreover, expressing the dominant-negative *RET* mutant *in vivo* in TT cells in nude mice led to prolonged survival, whereas inoculation of *ex vivo* transduced TT cells in nude mice led to complete suppression of tumor growth (303). Introduction of a mutant RET selective ribozyme (a RNA molecule capable of sequence-specific cleavage of other RNA molecules) that specifically cleaves mutant RET RNA and blocks RET-mediated cell growth and transformation may be another gene therapeutic approach (304).

Using adenoviral or retroviral vectors, small double-stranded RNA molecules (small interfering RNA) induce specific degradation of mRNA through complementary base pairing. Consequently, small interfering RNA molecules silence gene expression in mammalian cell culture as well as in animal models (305). Moreover, it seems that RNA interference (RNAi) can also serve as a tool to down-regulate chimeric fusion transcripts (305). RNAi offers a new way to inactivate genes of interest. It would be appealing to test the activity of RNAi and ribozyme-based gene therapy on clinically relevant models of RET-associated tumors.

The main challenge to any gene therapy, however, is to reach the tumor cells efficiently (71). The inhibition of oncogenic RET expression requires high levels of *in vivo* transduction efficiency, thereby limiting its therapeutic efficacy (302).

IX. Conclusion

In 20 yr, the critical role of *RET* in the growth of endocrine tumors has been well recognized. Soon after the discovery that the *RET* gene was responsible for MEN 2 and FMTC, genetic testing to treat patients with prophylactic thyroidectomy was applied, and recently, mutation-based treatment recommendations have been described. Despite the clear genotype-phenotype correlation in MEN 2, the molecular mechanisms linking the receptor with the (variable) disease phenotypes remain to be unraveled. In contrast to PTC, which has good adjuvant treatment, surgery remains the

only treatment with curative intent in MEN 2 related tumors, FMTC, and sporadic MTC and pheochromocytoma. Therefore, new treatment options are needed for patients with disseminated PTC and for patients with disseminated MTC or pheochromocytoma. Although numerous studies provided evidence that RET is a potential target for selective cancer therapy, a clinically useful therapeutic option for treating patients with *RET*-associated cancer is still not available.

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Address all correspondence and requests for reprints to: R. M. W. Hofstra, Ph.D., Department of Genetics, University Medical Center Groningen, University of Groningen, Hanzeplein 1, Postbus 30 001, 9700 RB Groningen. E-mail: r.m.w.hofstra@medgen.umcg.nl

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