Targeting the RET Pathway in Thyroid Cancer
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Abstract
The RET (rearranged during transfection) protooncogene encodes a single pass transmembrane receptor that is expressed in cells derived from the neural crest and the urogenital tract. As part of a cell-surface complex, RET binds glial derived neurotrophic factor (GDNF) ligands in conjunction with GDNF-family α co-receptors (GFRαs). Ligand-induced activation induces dimerization and tyrosine phosphorylation of the RET receptor with downstream activation of several signal transduction pathways. Activating germ-line RET mutations play a central role in the development of the multiple endocrine neoplasia (MEN) syndromes MEN2A, MEN2B, and familial medullary thyroid carcinoma (FMTC) and also in the development of the congenital abnormality Hirschsprung’s disease. Approximately 50% of patients with sporadic MTC have somatic RET mutations, and a significant portion of papillary thyroid carcinomas result from chromosomal inversions or translocations, which activate RET (RET/PTC oncoproteins). The RET protooncogene has a significant place in cancer prevention and treatment. Timely thyroidectomy in kindred members who have inherited a mutated RET allele, characteristic of MEN2A, MEN2B, or FMTC, can prevent MTC, the most common cause of death in these syndromes. Also, recently developed molecular therapeutics that target the RET pathway have shown activity in clinical trials of patients with advanced MTC, a disease for which there has been no effective therapy. (Clin Cancer Res 2009;15(23):7119–23)

Background
Medullary thyroid carcinomas (MTC) are derived from the neural crest C cells, not from the follicular cells, as are papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC). The malignancy presents either sporadically (75% of cases) or in a hereditary pattern (25% of cases) as either multiple endocrine neoplasia (MEN) type 2A, MEN2B, or familial MTC (FMTC). Clinically, the hereditary forms of MTC are characterized by complete penetrance but variable expressivity, such that the MTC occurs in virtually all patients, however, only 50% of patients with MEN2A and MEN2B develop pheochromocytomas, and 30% of patients with MEN2A develop parathyroid hyperplasia. Patients with FMTC only develop MTC (1).

The RET (rearranged during transfection) protooncogene (Fig. 1) is located in the pericentromeric region of chromosome 10q11.2 and spans 21 exons (2). RET encodes a receptor tyrosine kinase, which is expressed in neuroendocrine cells (including thyroid C cells and adrenal medullary cells), neural cells (including parasympathetic and sympathetic ganglion cells), urogenital tract cells, and testis germ cells. RET protein is structured with an extracellular portion (which contains four cadherin-like repeats, a calcium binding site, and a cysteine-rich region), a transmembrane portion, and an intracellular portion, which contains two tyrosine kinase subdomains (TK1 and TK2) that are involved in the activation of several intracellular signal transduction pathways. Alternate splicing of RET produces three isoforms with either 9, 43, or 51 amino acids at the C terminus, referred to as RET9, RET43, or RET51 (3).

A tripartite cell-surface complex is necessary for RET signaling. One of four glial derived neurotrophic factor (GDNF) family ligands (GFL), GDNF, neurturin, artemin, or persephin, binds RET in conjunction with one of four glycosylphosphatidylinositol-anchored co-receptors, designated GDNF-family α receptors (GFRαs): GFRα1, GFRα2, GFRα3, and GFRα4 (4–8). GDNF primarily associates with GFRα1, whereas neurturin, artemin, and persephin preferentially bind GFRα2, GFRα3, or GFRα4, respectively. Mice lacking GFRα4 have defects in calcitonin production, highlighting a role for the persephin receptor in regulating thyroid C cell function (9). Ligand stimulation leads to activation of the RET receptor with dimerization and subsequent autophosphorylation of intracellular tyrosine residues, which serve as docking sites for various adaptor proteins (10, 11).

RET is a versatile kinase, which is capable of directly phosphorylating multiple downstream targets (Fig. 1). Y905 is a binding site for Grb7/10 adaptors, however, the function of...
the adaptors has not been completely elucidated. The transcription factor STAT3 is activated through phosphorylation of Y752 and Y928. Janus kinases (JAK), c-Src, and the Ras/ERK pathway have also been involved in STAT3 activation by different RET-derived oncoproteins (12). Phosphorylated Y981 is the major binding site for Src, which is essential for neuronal survival. Src is also necessary for RET signaling through focal adhesion kinase (FAK), an important mediator of tumor cell migration and metastases (13). RET 981 is also involved in AKT activation (14). RET phospholipase Cγ interacts with activated RET by binding Y1015, thereby activating protein kinase C enzymes, which in turn are key regulators of receptor tyrosine kinases (15). Mice lacking RET 1015 have defects in kidney development (16). Nonphosphotyrosine dependent mechanisms are involved in RET signaling as well. Phosphorylation of RET S697 is critical for activation of the RAC1/JUN NH(2)-terminal kinase (JNK) pathway and mice lacking S697 exhibit enteric nervous system defects (17). The C-tail of RET9 but not RET51 binds the PDZ-containing scaffold protein Shank3, contributing to sustained ERK and PI3K signaling (18).

Finally, Y1062 is a highly important multidocking binding site for such proteins as DOK1/4/5, Enigma, FRS2, IRS1/2,
Shc, and ShcC. Phosphorylation of 1062 also results in activation of major intracellular pathways, including RAS/ERK, PI3K/ AKT, nuclear factor κB (NF-κB), and JNK (10, 12, 19–25). The well-characterized RAS > RAF > MEK > ERK pathway plays an important role in thyroid carcinoma as RAS mutations are found in FTC and in the follicular variant of PTC. BRAF mutations are found in PTC and anaplastic thyroid carcinoma. The role of RAS mutations in MTC is controversial (26, 27). The PI3K > AKT pathway plays a key role in RET signaling, however, to date there has been little genetic analysis of this pathway in MTC.

Recently, the biochemical characterization and structure of the human RET tyrosine kinase domain was reported to show that both the phosphorylated and nonphosphorylated forms adopt the same active kinase conformation necessary to bind ATP and substrate and have a pre-organized activation loop conformation that is independent of phosphorylation status (28).

### Clinical-Translational Advances

In 1990 Grieco and associates reported that some PTCs were caused by a transforming oncogene, resulting from the rearrangement of a heterologous amino terminal sequence to the tyrosine kinase domain of RET (29). It was subsequently shown that this hybrid oncogene, termed RET/PTC, was present in approximately 5 to 40% of PTCs and resulted from double-stranded DNA breaks with erroneous rearrangements involving the C-terminal portion of RET and the promoter and coding region of the N terminus of a constitutively expressed unrelated gene from chromosome 10 or a different chromosome (30). These fusion oncogenes were found to be particularly common in patients previously exposed to radiation, such as the children living near the Chernobyl nuclear accident, in patients exposed to external irradiation, or in victims of the atomic bomb explosion in Japan during World War II (31–33). Spatial proximity of RET and fusion partner's loci involved in the recombination in interphase chromatin has been shown as a mechanism of radiation-induced recombination (34). Most PTCs, however, are caused by an activating mutation in the B isoform of Raf kinase, BRAFV600E (35). PTCs caused by BRAF mutations, compared with those resulting from RET/PTC translocations, are larger, have a higher incidence of local tissue invasion and lymph node metastases, and have a poorer clinical prognosis (36).

In 1993 and 1994 it was shown that point mutations in the RET protooncogene cause MEN2A, MEN2B, and FMTC (see COSMIC catalog3 and the MEN2 database4 refs. 37, 38). MEN2A is associated with mutations involving the extracellular cysteine codons 609, 611, 618, 620 (exon 10) 630, or 634 (exon 11). The mutations associated with FMTC involve a broad range of codons including some associated with MEN2A, particularly, 609, 618, and 620, as well as others: 768, 790, and 791 (exon 13), 804 and 844 (exon 14), or 891 (exon 15). In 95% of patients with MEN2B there is a point mutation in codon 918 (exon 16, Met918Thr) within the intracellular domain of RET (39). A few patients with MEN2B have a mutation in codon 883 (exon 15; ref. 40). Single MEN2B patients with double RET mutations Val804Met + Ser904Cys and Val804Met + Tyr806Cys have been reported (41, 42). In MEN2A and FMTC the RET mutations lead to a ligand-independent homodimerization and constitutive kinase activity such that RET becomes a dominant oncogene. In MEN2B RET mutations activate the RET receptor in its monomeric state, leading to phosphorylation of Y1062 and other tyrosines, and also causing a change in substrate specificity (9).

It is likely that RET does not act alone in the genesis of hereditary MTC, and studies of MTC tissues and cell lines from patients with MEN2A have shown evidence of a second hit as evidenced by trisomy 10 with duplication of the mutant RET allele, loss of the wild-type RET allele, and a tandem duplication event with amplification of mutant RET (43). Almost certainly additional genetic defects, such as chromosomal deletions and amplifications will be shown in future studies. Probably, given the young age at presentation, fewer additional genetic alterations occur in MTC in patients with the germline MEN2B mutation. A recent array CGH study unveiled many gene copy number alterations in MTC patients (44). Approximately 50% of patients with sporadic MTC have somatic mutations in RET codon 918 and these tumors seem to be more aggressive clinically compared with tumors lacking the somatic RET mutation (45).

In patients with MEN2A, MEN2B, and FMTC there is a correlation between genotype and phenotype, both in the clinical expression of the disease spectrum and the clinical aggressiveness of MTC. Whereas, virtually all patients develop MTC, pheochromocytomas develop in approximately 50% of patients with MEN2A and MEN2B. In MEN2A they occur most often in patients with RET mutations in codon 634 and less frequently in patients with mutations in codons 618, 620, or 791. Hyperparathyroidism occurs only in patients with MEN2A who have mutations in codons 618, 630, 634, or 791. Considering the age of onset and clinical aggressiveness of MTC, patients are classified as being at highest risk, higher risk, or high risk. Patients with MEN2B, who have mutations in codons 918 or 883 represent the highest risk group, whereas patients with mutations in codons 634, 630, 609, 611, 618, and 620 are in the higher risk group. The high risk group includes patients with mutations in codon 768, 790, 791, 804, and 891 (46).

Rarely, the dermatologic disorder cutaneous lichen amyloidosis and the congenital disorder Hirschsprung's disease (HSCR; aganglionic megacolon) may occur in association with MEN2A (46, 47). Hirschsprung's disease, the most common cause of intestinal obstruction in the newborn, is characterized by the absence of enteric ganglia in variable segments of intestine. A RET mutation has been identified in only 50% of familial and 15 to 20% of sporadic cases of HSCR (48, 49). More than 100 different RET mutations have been described, and, in contrast to mutations in MEN2A, MEN2B, FMTC, and sporadic MTC, which are virtually all missense mutations, they include microdeletions, insertions, nonsense and missense, and splicing mutations. In some cases the entire RET gene is deleted (50). The RET mutations associated with MEN2A, MEN2B, and FMTC syndromes are generally gain-of-function mutations, whereas the RET mutations occurring in HSCR are loss-of-function mutations, most likely associated with a haploinsufficiency or dominant negative effect.

3 http://www.sanger.ac.uk
4 http://arup.utah.edu/database/MEN2/MEN2_welcome.php
Rarely, patients with MEN2A who have RET mutations in codons 609, 611, 618, or 620 (exon 10) develop HSCR (47). Functional studies have shown that cell surface expression of these RET mutants is lower than that associated with other RET mutations, such as 634 (51). Thus, these four mutations lead to constitutive activation of RET and continued proliferation of C cells on one hand, whereas on the other, they result in a marked reduction of RET expression at the cell membrane, and a resultant apoptosis of enteric neurons.

There is no better example of the translation of genetic information into clinical practice than that provided by detection of RET mutations in patients with hereditary MTC. Shortly, after the discovery that MEN2A, MEN2B, and FMTC were caused by mutations in the RET protooncogene, it became clear that family members who carried a mutated RET allele could be detected by direct DNA analysis. Knowing that the MTC was the most common cause of death in patients with these syndromes and that the thyroid gland was an expendable organ whose function could be easily replaced by the oral administration of the drug thyroxin, several centers initiated screening programs in MEN type 2 families to identify kindred members with RET mutations. The thyroid gland can be removed safely and the operation is curative if done at a young age, before the MTC develops or is still confined to the thyroid gland (52, 53). Although, there is general agreement that prophylactic thyroidectomy is indicated in members of families with hereditary MTC, the timing of thyroidectomy depends on the site of the RET codon mutation. Thyroidectomy is advised at a very early age (even in the first months of life) in patients at highest risk (mutations in codons 918 or 883), at 5 years of age in patients at higher risk (mutations in codons 611, 618, 620, and 634), and between 5 and 10 years of age in patients at high risk (mutations in codons 609, 768, 790, 791, 804, or 891; refs. 54, 55).

The finding that the molecular targeted therapeutic (MITT) STI571 (imatinib mesylate, Novartis AG) induced remissions in patients with chronic myelogenous leukemia has had a profound effect on clinical medicine and has changed the landscape of cancer therapy (56). The anilinoquinazoline vandetanib (AstraZeneca Limited) selectively inhibits the kinase insert domain-containing receptor [KDR/vascular endothelial growth factor receptor (VEGFR2) tyrosine kinase activity (IC_{50} \sim 40 \text{nM})]. The compound also has activity against VEGFR3; IC_{50} = 110 \text{nM} and epidermal growth factor receptor (EGFR/HER1; IC_{50} = 500 \text{nM}; ref. 57). In 2002 Carломagno and associates showed that ZD6474 is a potent inhibitor (IC_{50} = 100 \text{nM}) of RET oncoproteins. These investigators also found that ZD6474 has a marked inhibitory effect on the growth of thyroid cancer cells with spontaneous RET/PTC rearrangements and also the growth of NIH-RET/PTC xenografts (58). Phase I studies showed that ZD6474 administration is associated with minimal toxicity and subsequent phase II clinical trials showed that ZD6474 has activity in patients with locally advanced or metastatic hereditary MTC. Approximately 20% of patients experienced a partial remission, as shown by RECIST (response evaluation criteria in solid tumors) criteria and another 60% experienced stable disease for a disease control rate of 80% (59). Following treatment, there was reduction in serum levels of calcitonin and carcinoembryonic antigen, tumor markers secreted by the MTC cells. Subsequently, it was shown that vandetanib induced confirmed partial remissions in 85% of children with advanced MEN2B (60). Phase II clinical trials of other MITTs have also shown activity in patients with advanced MTC (61, 62).

The molecular analysis of tumor tissue in patients whose chronic myeloid leukemia recurred following initial treatment with imatinib mesylate was fundamental to the design of successful rescue therapy and to the development of combinatorial regimens to prevent disease recurrence following primary treatment (63). Unfortunately, there has been a paucity of such studies in clinical trials of MITTs in advanced thyroid cancer, primarily because tissue acquisition has been infrequent or non-existent. This issue must be corrected if we are to improve the treatment of patients with this disease.

Disclosure of Potential Conflicts of Interest

S.A. Wells, Jr., consultant, AstraZeneca. M. Santoro, commercial research grant, AstraZeneca.

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