Molecular Pathways

Targeting RET Receptor Tyrosine Kinase Activation in Cancer

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Abstract

After ligand binding induces dimerization, the RET receptor tyrosine kinase activates multiple signal transduction pathways. Constitutively activating mutations and chromosomal rearrangements are the primary oncogenic event in a significant number of medullary thyroid cancers (MTC) and papillary thyroid cancers (PTC), respectively. When specific germ-line mutations in RET are identified early, prophylactic thyroidectomy can be timed to remove at-risk tissue in patients with multiple endocrine neoplasia 2 (MEN2) syndromes who would otherwise develop MTC. Conventional therapy for progressive metastatic MTC is limited. Small-molecule tyrosine kinase inhibitors can target multiple kinases at nanomolar concentrations including RET and have shown efficacy against a variety of malignancies. Initial clinical evidence suggests that several of these including Sorafenib, Vandetanib, Motesanib, Sunitinib, and XL-184 may have some benefit in treating progressive MTC. While initial success seen in these trials appears to be modest, this represents a major breakthrough in treatment of patients with widespread metastatic MTC.
Background

Receptor tyrosine kinases (RTK) constitute a large family of receptors which in response to their ligand activation are potent mediators of cell motility, proliferation, differentiation, and survival. Dysregulation of RTK signaling is one of the most common molecular defects associated with malignancy. The RET receptor protein was one of the first RTK found to play a role in neoplasia. The protein is encoded by the REarranged during Transfection (RET) proto-oncogene on chromosome 10q11.2. Over 20 years ago, the gene was shown to be associated with papillary thyroid carcinoma (PTC) through chromosomal rearrangements (RET/PTC) (1). In 1993-94, point mutations in the RET proto-oncogene were determined to be responsible for virtually all inherited medullary thyroid cancer (MTC) (2, 3). Furthermore, point mutations in the RET gene are found in up to 50% of sporadic MTC. While both of these alterations lead to a gain-of-function and subsequent tumorigenesis, Hirschsprung’s disease (loss of enteric neurons) is associated with loss-of-function germ-line mutations. There are two significant RET isoforms from alternative splicing, resulting in different lengths of the carboxy 3’ terminal region: RET9, RET51. These isoforms are coexpressed in most tissues, but do not form heterodimers in vivo. The two isoforms have distinct developmental roles, and different gene expression profiles on microarray analysis suggest possible differences in downstream regulation of cell-cell interactions (4).

The RET protein is composed of three domains: an extracellular ligand binding domain, a transmembrane domain, and a cytoplasmic tyrosine kinase domain. The extracellular domain contains four cadherin-like repeats as well as a highly conserved cysteine-rich region. The cysteine-rich region is important for tertiary structure and dimerization through disulfide bond formation. The ligands of the RET receptor were identified in 1996 as growth factors belonging to the glial cell-line derived neurotrophic factor (GDNF) family (5-7). The GDNF-family ligands (GFL) include GDNF, neurturin (NRTN), artemin (ARTN), and persephin (PSPN). RET activation requires formation of a multimeric complex with a ligand, a GDNF-family receptor-α (GFRα) protein, and RET (Fig. 1). GFRα are glycosylphosphatidylinositol (GPI)-anchored co-receptors with no transmembrane or intracellular domain. Four GFRα receptors (GFRα1-4) have been
identified which preferentially bind the different GFL’s. The GFL and GFRα association leads to RET dimerization to form a GFL(2)-GFRα(2)-RET(2) heterohexamer complex that results in intracellular kinase activation and signaling. GDNF and GFRα1 knockout mice display a similar phenotype to RET knockout mice, namely lack of enteric neurons and kidney agenesis. NRTN and GFRα2 knockout mice lack parasympathetic cholinergic innervation in the salivary glands. ARTN and GFRα3 knockout mice have a reduction or lack the superior cervical ganglion. PSPN and GFRα4 knockout mice demonstrate hypersensitive cerebral ischemia and decreased calcitonin secretion respectively (8).

Lipid-rafts on the plasma membrane have also been shown to be important for RET signal transduction and likely interact with the activating complex in two different ways. GFRα receptors are typically located in lipid rafts, and after ligand binding recruit RET into the raft where it dimerizes and interacts with specific docking proteins which activate signal pathways. Phosphorylated RET can then move away from the lipid rafts where it can associate with different docking proteins and is possibly degraded or internalized. Alternatively, GFRα may be cleaved from the plasma membrane to produce a soluble form, which may interact with GFL before associating with RET which then is recruited to the lipid-raft.

RET protein dimerization results in autophosphorylation of several intracellular RET tyrosine residues. There are 10 autophosphorylation sites found on both major RET isoforms (RET9 and RET51), and an additional two found on the longer isoform, RET51. Several are binding sites for a variety of docking proteins. The tyrosine(Y) Y1062 has been shown to bind SHC (Src-homology collagen), IRS1/2 (insulin receptor substrate1/2), FRS2 (fibroblast growth factor receptor substrate 2), and PKCα (protein kinase Cα). These are able to activate multiple signaling pathways, including MAPKinase (mitogen activated protein kinase), PI-3K(phosphatidylinositol-3-kinase)/AKT, RAS/ERK, and Rac/JNK. These pathways are mediators of cell motility, proliferation, differentiation, and survival. DOK 1/4/5/6 (downstream of kinase 1/4/5/6) also binds phosphorylated Y1062, and DOK4 binding has been implicated in GDNF-dependent outgrowth. Binding of c-Src or SH-2Bβ to phosphorylated Y981 promotes survival and differentiation. Other binding sites as well have been shown to be
important. SHC preferentially binds to activated RET outside lipid-rafts, while FRS2 (FGF receptor substrate 2) preferentially binds when RET is within the raft. FRS2 activates ERK through both Grb2 and Shp2.

**Phenotype-Genotype Correlation in Medullary Thyroid Cancer**

MTC arises from the calcitonin-producing thyroid C cells. One quarter of all MTC arise in an inherited form from point mutations in the RET proto-oncogene. These familial syndromes include multiple endocrine neoplasia type 2A (MEN2A), type 2B (MEN2B), and familial MTC (FMTC), in which the penetrance of developing MTC is virtually 100%. The RET gene mutations have a high phenotype-genotype correlation, corresponding with MTC behavior and directly affecting treatment and surveillance. Generally, the least aggressive tumors arise in FMTC, which is characterized by only the development of MTC without other abnormalities. MEN2A tumors are slightly more aggressive, and patients may develop pheochromocytomas, parathyroid hyperplasia, and rarely cutaneous lichen amyloidosis. MEN2B tumors are the most aggressive, and the syndrome is characterized by pheochromocytomas, skeletal abnormalities, mucosal neuromas, and a Marfanoid habitus but not parathyroid hyperplasia. Presently, there are 500 - 1000 MEN2 kindreds recognized worldwide (9).

RET mutations in MEN 2A kindreds fall predominantly into one of six cysteine residues in the extracellular domain (codons 609, 611, 618, 620, 630, and 634). Of these, mutations in codon 634 account for approximately 85%. These cysteine residues are essential for the protein tertiary structure, as they form intramolecular disulfide bonds. When one of these six cysteines is mutated, then an unpaired cysteine is left, which is believed to be able to form a disulfide bond with another similar RET protein, resulting in dimerization and activation without ligand stimulation. There are also rare reports of kindreds with a short 9 or 12 base pair insertion disrupting a cysteine residue, again resulting in an unpaired cysteine available for dimerization. Rare MEN2A mutations exist in non-cysteine codons, including 533, 790, 791, 804, and 891. The last four mutations occur in the intracellular tyrosine kinase domain. The penetrance of hyperparathyroidism in MEN2A is about 20% and is more commonly associated with codon 634 mutations. Pheochromocytomas, seen in approximately 50% of patients, are
most frequently associated with codon 634, but have been reported with all codons except 630. Cutaneous lichen amyloidos is also has rarely been reported with MEN2A and has only been described associated with codon 634. Surprisingly, MEN2A kindreds can also demonstrate a Hirschsprung’s phenotype (typically associated with a loss of function mutation), which has only been associated with codon 609, 611, 618, and 620 mutations. One explanation for this paradox is that in combination with this activating RET mutation there are multiple mutations affecting the RET gene or the upstream regulatory elements that occur within a specific haplotype (10). FMTC kindreds have germline mutations which overlap MEN2A mutations, but also include mutations from additional codons including 768, 844, and 912. RET mutations in MEN2B kindreds fall almost entirely at codon 918 (95%), but a few families have a codon 833 mutation. These mutations occur in the catalytic region of the intracellular tyrosine kinase domain, enabling activation without the need for ligand stimulation or RET dimerization. Several studies have suggested differences in substrate specificity between MEN2A and 2B activated proteins. MEN2B-mutated RET has a higher upregulation of PI-3K/AKT and JNK phosphorylation. A difference in the pattern of RET autophosphorylation sites has also been demonstrated. Several other rare MEN2B mutations have a mutation in codon 804 with a simultaneous mutation in codons 805, 806, or 904.

The only other malignancy besides thyroid where mutations in RET clearly contribute to malignancy is pheochromocytoma. Sporadic pheochromocytomas were found to contain a somatic, heterogenous 918 mutation in 15% of examined cases(11). Initially, it was reported that two small cell lung cancers had RET mutations, but this was not corroborated in larger studies. Mutations have not been detected in other neuroendocrine tumors, including neuroblastomas, which expresses wild type RET. Recently, there has been evidence that some pancreatic ductal adenocarcinomas contain a RET polymorphism. In a study of 52 primary tumors, the allelic frequency of a G619S polymorphism was 20%, while in matched normal pancreas the frequency was 15%(12). The G619S polymorphism may enhance GDNF receptor mediated cell proliferation and invasion, but a clear role in pancreatic cancer pathogenesis has yet to be demonstrated.
Clinical-Translational Advances

Genetic Determination of Surgical Timing

The optimal treatment for MTC in patients with MEN2 is prophylactic thyroidectomy, ideally just prior to extra-thyroidal spread. Since there is a good correlation between MTC clinical aggressiveness and the specific RET genotype, the timing of surgical intervention varies depending on the specific mutation. The American Thyroid Association has recently refined the categorization of all known mutations into four levels to recommend an age for prophylactic surgery (13). Patients with the highest risk are in level D with mutations in codons corresponding to MEN2B, and should have surgery by age 6 months. Level C consists of mutations in codon 634 and should have prophylactic surgery before age 5. Level B consists of mutations in codons 609, 611, 618, 620, and 630. Surgery should be considered before age 5, but may be delayed if stringent criteria are met (normal serum calcitonin, normal neck ultrasound, and less aggressive family MTC history). Level A mutations are characterized by MTC with the least aggressive behavior and surgery may be delayed after age 5 based on stringent criteria previously described and the clinicians discretion.

Targeted Receptor Tyrosine Kinase Inhibition

Over the past decade has come the development of small-molecule tyrosine kinase inhibitors (TKIs), which typically affect multiple signaling pathways. Currently, an inhibitor specific only for RET is not available, but several multi-kinase inhibitors have significant activity against RET. Several have demonstrated inhibition of RET kinase and tumor growth in pre-clinical models of MTC. Vandetanib (ZD6474) was originally developed as a second generation epidermal growth factor receptor (EGFR) TKI but subsequently was found to have more potent inhibitory effects against vascular endothelial growth factor receptor (VEGFR)(IC$_{50}$ = 40 nM) and RET (IC$_{50}$ = 130 nM) than EGFR (IC$_{50}$ = 500 nM) (14). Vandetanib blocks autophosphorylation of codon 918 mutant RET kinase in intact cells (15). Certain mutations in RET codons 804 and 806 have been shown to confer resistance to vandetanib, which may be a concern for secondary resistance to the drug (16, 17). Sorafenib (BAY 43-9006) is another multi-
kinase inhibitor targeting RET, as well as BRAF, VEGFR, and platelet-derived growth factor receptor (PDGFR). In vitro, sorafenib inhibits oncogenic RET kinase with an IC$_{50}$ of $<50$ nM and decreased tumor volume of TT cells (MTC cell line harboring a codon 634 RET mutation) in athymic mice (18).

Phase I and II clinical trials of at least 15 patients evaluating efficacy of RET TKI’s in patients with metastatic MTC are summarized in Table 1. The phase II trial of vandetanib was conducted exclusively in patients with hereditary MTC (19). While it demonstrated dramatic suppression of serum calcitonin and CEA in 80% of patients, partial responses induced were modest (20%). Responses did not correlate with 618, 620, 634 or 918 codon mutations in RET. The phase II trials for motesanib and sorafenib included patients with sporadic as well as hereditary MTC (20, 21). Although these trials included mostly sporadic MTC, somatic mutations in RET were present in 70-80% of tumors tested; and of these, greater than 80% were at codon 918. As indicated in Table 1, partial responses with motesanib and sorafenib were minimal (2-6%) and overall responses were modest at best. Finally, data for sunitinib and XL-184 are preliminary but reveal modest partial responses (29-33%) and tumor marker responses (22-24). Like the sorafenib and motesanib trials, the RET mutation rate in sporadic tumors was also high (65-78%) with predominantly codon 918 mutations.

Based on encouraging response and safety data found in early phase clinical trials in MTC, an international multicenter double blind randomized phase III clinical trial in MTC (hereditary and sporadic combined) with vandetanib has been conducted while another with XL-184 is ongoing. While both trials use placebo as a control group with 2:1 randomization, unblinding and cross over at time of disease progression was allowed in the vandetanib trial but not in XL-184. The primary endpoint is progression free survival (PFS) in the vandetanib trial while overall survival is used in the XL-184 trial. Preliminary results of phase III trial of vandetanib were reported after 24 months of median duration of follow up (25). A total of 331 patients were enrolled from December 2006-November 2007, with 231 patients assigned to receive oral vandetanib at a dose of 300 mg daily on an ongoing basis while 100 patients were assigned to receive placebo. Median progression free survival for the placebo group was 19.3 months while it has not been reached for vandetanib group. The hazard ratio for progression free survival is 0.46
(95% CI: 0.31-0.69) with p-value of <0.0001. No unexpected adverse events were noted. This is the first phase III trial in sporadic and hereditary MTC showing benefit of RET-targeted TKI in delaying disease progression. However, overall survival data is immature.

Common grade 1-2 (mild-moderate) adverse events such as fatigue, weight loss, diarrhea, mucositis, hand foot syndrome, hypertension are observed with TKI therapies and can adversely affect quality of life. Furthermore, grade 3-4 (severe and life threatening) side effects such as bowel perforation, thrombosis/bleeding are uncommon but do occur.

In summary, trials of vandetanib, motesanib, sorafenib, sunitinib and XL-184 seem to be within an appropriate dose and in an appropriate patient population for targeting RET kinase inhibition. All of these kinase inhibitors except imatinib exhibit very low IC$_{50}$ (4-130 nM) for RET kinase that is easily achievable in patients’ plasma with standard oral doses of these drugs. While trials of motesanib, sorafenib, sunitinib and XL-184 did not require a positive RET mutation as entry criteria, the sporadic MTCs in these trials were enriched for RET mutation tumors. Although direct comparisons between these trials cannot be made, a few observations can. Responses are observed across these trials but response rates are variable which may relate to differences in tumor biology, host or drug characteristics. One or more of the following factors may explain the failure to achieve higher and durable responses despite using an appropriate drug and patient population in these trials: 1) degree of tumor burden and pace of progression, 2) frequency of codon 918 RET mutation (associated with aggressive clinical course), 3) pharmacogenomics, 4) potency and duration of RET kinase inhibition by kinase inhibitor, 5) target profile of multi-kinase inhibitor, and 6) drug tolerability impacting dose adjustment and drug holidays. In order to optimize response rates and toxicity profiles, actual targets and mechanism of action as well as mechanisms of primary and secondary resistance need clarification. Presently, it is unknown if observed responses are related to RET kinase inhibition, other kinases inhibited by these drugs, or a combination of both. VEGFRs inhibition may play a critical role in inducing responses by such multi-kinase inhibitors.
Overall success seen in these trials represents a major breakthrough in treatment of patients with widespread metastatic MTC. However, current clinical trials of RET-targeted therapies are only the first step in discovering effective therapies for patients with MTC. Further progress in understanding the molecular pathogenesis of MTC is critical to elucidate: 1) role of RET kinase signaling pathway in tumor progression and maintenance, 2) other critical targets or signaling pathways important in MTC, 3) mechanisms of primary and secondary resistance to TKIs by potential redundant signaling pathways or by developing ‘resistance’ mutations in RET. Combination or sequential targeted therapies based on strong pre-clinical data of inhibition of parallel or different signaling pathways may improve effectiveness. In addition, refining toxicity profile of targeted therapies is also a critical goal. Improving success rates of targeted therapies for MTC are realistic goals for near future in field of MTC.
References

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Figure Legend

Figure 1. Schematic RET protein structure showing common mutation and phosphorylation sites. RET forms a heterocomplex with GDNF-family ligands (GFLs) and GDNF-family receptor-α (GFRα) proteins to activate intracellular kinase activity. This results in activation of multiple signaling pathways important for survival, differentiation, motility, proliferation, and growth.
Table 1. Summary of Clinical Trials using Multi-Kinase Inhibitors in Medullary Thyroid Carcinoma

<table>
<thead>
<tr>
<th>Name of the drug tested</th>
<th>Imatinib</th>
<th>Vandetanib</th>
<th>Motesanib</th>
<th>Sorafenib</th>
<th>Sunitinib</th>
<th>XL-184</th>
</tr>
</thead>
<tbody>
<tr>
<td>RET IC50 (nM)</td>
<td>25,000</td>
<td>130</td>
<td>59</td>
<td>50</td>
<td>224</td>
<td>4</td>
</tr>
<tr>
<td>Key Targets besides RET</td>
<td>PDGFR, KIT</td>
<td>VEGFRs, EGFR</td>
<td>VEGFRs, PDGFR, KIT</td>
<td>VEGFRs, PDGFR, BRAF, KIT</td>
<td>VEGFRs, PDGFR, KIT</td>
<td>VEGFRs, c-MET, KIT</td>
</tr>
<tr>
<td>Phase of the trial</td>
<td>Phase II</td>
<td>Phase II</td>
<td>Phase II</td>
<td>Phase II</td>
<td>Phase II</td>
<td>Phase I/II</td>
</tr>
<tr>
<td>Period of accrual</td>
<td>Jan 03-Jan 05</td>
<td>Nov 04-Aug 06</td>
<td>Jul 05-Mar 06</td>
<td>Nov 06-Jan 08</td>
<td>Nov 06-Aug 09</td>
<td>Unknown-Aug 08</td>
</tr>
<tr>
<td>Oral drug dose</td>
<td>600 mg PO qd ongoing</td>
<td>300 mg PO qd ongoing</td>
<td>125 mg PO qd x 48 weeks</td>
<td>400 mg PO BID ongoing</td>
<td>50 mg qd x 4 wks on-2 wks off cycle; ongoing</td>
<td>75 mg to 175 mg qd ongoing</td>
</tr>
<tr>
<td>No. of pts (sporadic/Hereditary/unknown)</td>
<td>15 (11/4/0)</td>
<td>30 (0/30/0)</td>
<td>91 (76/13/2)</td>
<td>21 (16/5/0)</td>
<td>25 (14/2/9)</td>
<td>37 (3/28/6)</td>
</tr>
<tr>
<td><em>No. of pts with RET+ genotype/no. of pts tested (codon of RET mutation x no. pts)</em></td>
<td>3/3 sporadic (918 x 2, 883 x 1)</td>
<td>N/A sporadic</td>
<td>28/39 sporadic (918 x 25, other x3)</td>
<td>10/12 sporadic (918 x 9, 634 x 1)</td>
<td>9/14 sporadic (918 x 8, 634 x 1)</td>
<td>22/28 sporadic (918 x 15, 634 x 2, 620 x 1, other x 4)</td>
</tr>
<tr>
<td>Partial Response n (%)</td>
<td>0</td>
<td>6 (20)</td>
<td>2 (2)</td>
<td>1 (6)</td>
<td>8 (33)</td>
<td>10 (29)</td>
</tr>
<tr>
<td>Stable disease &gt;6 months n (%)</td>
<td>4 (27)</td>
<td>16 (53)</td>
<td>44 (48)</td>
<td>10 (62)</td>
<td>11 (46)</td>
<td>15 (41)</td>
</tr>
<tr>
<td>Median progression free survival (months)</td>
<td>Not reported</td>
<td>28</td>
<td>12</td>
<td>18</td>
<td>12</td>
<td>Not reported</td>
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<tr>
<td>≥50% decline in serum calcitonin compared to baseline % of pts</td>
<td>Not reported</td>
<td>80%</td>
<td>37%</td>
<td>42%</td>
<td>50%</td>
<td>72% (16 of 19 assessable pts)</td>
</tr>
</tbody>
</table>

£: Results of phase III trial of vandetanib vs placebo are only discussed in the text.
*: RET genetic testing was done in blood for pts in hereditary MTC while was done in the tumors for pts with sporadic MTC.
†: Responses, progression free survival and calcitonin response data are reported for 16 pts with sporadic MTC on this trial.
§: Preliminary results. Of 37 pts on XL-184 phase I/II, 13 pts were enrolled on dose escalation cohort while 24 pts were enrolled on expanded MTC phase II cohort.
¥: Progressive disease as defined by RECIST in 6 months prior to study entry or biochemical PD (for sunitinib study)/symptomatic disease (motesanib study) was required as at study entry.
€: Partial response is defined per RECIST (≥ 30% reduction in sum of diameter of tumor index lesions)
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