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Review

Molecular mechanisms of medullary thyroid carcinoma: current approaches in diagnosis and treatment

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Summary. Medullary thyroid carcinoma is the most common cause of death among patients with multiple endocrine neoplasia (MEN) 2. Dominant-activating mutations in the *RET* proto-oncogene have been shown to have a central role in the development of MEN 2 and sporadic medullary thyroid cancer (MTC): about half of sporadic MTCs are caused by somatic genetic changes of the RET oncogene. Inactivating mutations of the same gene lead to Hirschprung disease and other developmental defects. Thus, *RET* genetic changes lead to phenotypes that largely depend on their location in the gene and the function and timing of developmental expression of the RET protein. The reproducibility of the phenotype caused by each RET genotype led to MEN 2/MTC being among the first conditions in Medicine where a drastic measure is applied to prevent cancer, following genetic testing: thyroidectomy is currently routinely done in young children that are carriers of MTC-predisposing *RET* mutations. RET inhibitors have been also developed recently and are used in various types of thyroid and other cancers. This report reviews the RET involvement in the etiology of MEN 2 and MTC and updates the therapeutic approach in preclinical and clinical studies.

Key words: Multiple endocrine neoplasia type 22, Medullary thyroid cancer, *RET* oncogene, Tyrosine kinase inhibitors

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Introduction

Medullary thyroid carcinomas (MTC) are relatively rare thyroid tumors since they represent less than 10% of all thyroid malignancies (Sherman et al., 2005). However, unlike the situation in other human tumors, about 25 to 30% of all MTCs are heritable and caused by gain of function, germline mutations in the RET proto-oncogene (Fialkowski and Moley, 2006). MTCs, shortly after their first description (Hazard et al., 1959), were among the first human tumors to be reported in association with other neoplasms as a possible new syndrome as early as in 1961 by Dr. Sipple. In two reports that followed shortly thereafter (Schimke and Hartmann, 1965; Steiner et al., 1968), the genetic basis of "syndromic" MTCs and associated tumors was established, under the rubric "multiple endocrine neoplasia type 2" (MEN 2) (Steiner et al., 1968), to distinguish it from the condition described by Wermer, 1954 and known as multiple endocrine neoplasia type 1 (MEN 1). Familial MTC (FMTC) and/or inherited MTC, was characterized in the 1980s (Farndon et al., 1986). FMTC was found to be caused by mutations in the same gene (Eng, 1996; Eng et al., 1996), the RET oncogene, as the other two forms of MEN2: MEN2A and MEN2B. It should be noted that MEN2B is essentially a distinct syndrome with unique features (Williams and Pollock, 1966), a fact that also led to its multiple identifiers in genetic texts (MEN type 3 or Wagenmann-Froboese syndrome) (Fryns and Chrzanowska, 1988; Morrison and Nevin, 1996).

Pathology of MTC

MTC arises from the parafollicular or C-cells, known for the secretion of calcitonin. C-cells can be found anywhere in the thyroid gland but are mostly concentrated in the posterior upper third of the lateral aspect of both lobes (Cameron, 2004; Guillem et al., 2006; Ogilvie and Kebebew, 2006) These tumors are

slow growing and familial forms are usually multifocal and bilateral. The histology of MTC can be quite variable: Papillary or pseudopapillary MTC, characterized by the presence of true papillae or pseudopapillae (occasionally this may be caused by tissue fragmentation) is the most common type. Other types include glandular (tubular or follicular), giant, spindle, small or clear cell, paraganglioma-like, angiosarcoma-like, and even melanin-producing MTC.

Metastatic potential of MTC

Half of the patients may have nodal metastases at presentation; MTC frequently metastasizes to regional lymph nodes. Distant metastases may be present in as many as a sixth of the patients. Spread is most frequent to the central compartment, followed by the ipsilateral jugular chain of nodes and the contralateral cervical nodes. Less frequently, the tumors grow locally to the upper and anterior mediastinum and may impair esophageal or respiratory function, or they may produce enough calcitonin to cause diarrhea, flushing and other symptoms. More rarely, Cushing syndrome and other paraneoplastic conditions may develop from other peptides produced by the tumor. Hematogenous spread may occur to the lungs, liver, bones, brain and soft tissues. Prognosis of sporadic MTCs varies widely among patients (Peixoto et al., 2006).

Sporadic medullary thyroid carcinoma and the MEN 2 syndromes

Sporadic MTCs present on average at an age of 50 years, whereas inherited MTCs present earlier with the exception of FMTC (Gharib et al., 1992; Kebebew et al., 2000; Clayman and el-Baradie, 2003; Gulben et al., 2006). Patients with sporadic MTC usually present first with a palpable thyroid nodule. This tumor does not uptake radioactivity on scintigraphic scan (it is a “cold” nodule) (Gharib et al., 1992; Clayman and el-Baradie, 2003; Gulben et al., 2006). Although C-cell hyperplasia (CCH) precedes the development of inherited MTC, the role of CCH in sporadic MTC is less clear. Sporadic MTC some times is found in the context of Hashimoto’s thyroiditis and rats treated with low doses of ^{131}I develop MTC more frequently but generally the causes of these tumors are unknown.

Sporadic MTCs are caused by somatic *RET* point mutations and occasional deletions in approximately 40–50% of the cases (Blaugrund et al., 1994; Hofstra et al., 1994; Zedenius et al., 1994; Eng et al., 1995; Marsh et al., 1996; Romei et al., 1996; Wohllk et al., 1996; Kebebew et al., 2000). The role of relatively frequent germline *RET* gene variants in the possible predisposition to MTC of patients without any family history is more controversial (Grisieri et al., 2000; Ruiz et al., 2001; Elisei et al., 2004) The molecular mechanisms leading to sporadic MTC (and the modes of treatment, therefore) are not different from those for inherited MTCs and they are being discussed below.

As already mentioned, the first among the multiple endocrine neoplasias (MEN) syndromes that was molecularly elucidated was the group of conditions known as MEN type 2 (MEN 2) (Kebebew et al., 2000). There are at least 3, phenotypically distinct, genetic syndromes in this group: FMTC, MEN 2A and MEN2B. FMTC is not associated with any other tumors; MEN2A is a diagnosis that describes the association of MTC with hyperplasia and/or tumors of the parathyroid glands and the adrenal medulla (i.e. pheochromocytoma); finally, MEN2B, a very rare condition, is a genetic syndrome in which MTC, pheochromocytoma, mucosal neuromas, ganglioneuromas, and other neuronal tumors are present in individuals with a Marfanoid habitus (Williams and Pollock, 1966; Fryns and Chrzanowska, 1988; Morrison and Nevin, 1996). All these conditions are caused by mutations in the *RET* oncogene.

Clinical presentation

MTC is the most common lesion of the MEN 2 syndromes (Clayman and el-Baradie, 2003; Gulben et al., 2006). Despite the presence of histologic evidence of MTC in more than 95% of obligate gene carriers by age 35, calcitonin hypersecretion is not as specific as was initially believed. Patients who have been identified on the basis of calcitonin provocative tests as not having MEN 2A or 2B have been shown to be obligate noncarriers of their kindred's characteristic *RET* mutation. Conversely, patients classified as being affected on the basis of CCH alone should be reevaluated with *RET* testing to allow accurate counseling about their children’s risk for MEN 2 (Eng, 1996; Eng et al., 1996).

Pheochromocytoma typically affects 50% to 60% of MEN 2A kindreds with a high rate of bilaterality, but a low rate of extra-adrenal sites or malignancy. The tumor is very common in MEN 2B and absent by definition in FMTC. Pheochromocytoma often does not present with hypertension or other typical symptoms which typically occur only in 50% of the patients (Lenders et al., 2005). MEN 2-related pheochromocytomas are characterized by production of epinephrine only or epinephrine together with norepinephrine and are therefore best detected by elevations of plasma or urinary metanephrine, usually but not always in association with elevations of normetanephrine and parent catecholamines. MEN 2-related pheochromocytomas are very rarely malignant (<5%) (Lenders et al., 2005). In addition, as with most epinephrine-secreting pheochromocytomas, hypertension when present is more likely to be paroxysmal than sustained (Zelinka et al., 2006). For these reasons the diagnosis is easy to miss. A yearly program for pheochromocytoma that uses plasma catecholamines and metabolites to screen can effectively identify tumors at an early stage (<3 cm), before the development of hypertension or other adverse sequelae. When a pheochromocytoma is diagnosed, laparoscopic

adrenalectomy affords a less invasive approach to most MEN 2-related such tumors; adrenal-sparing surgery may also have a role in the future (Asari et al., 2006).

Parathyroid disease is detected clinically in 10% to 15% of patients with MEN 2A (Herfarth et al., 1996; Vierhapper et al., 2005). Two distinct clinical variants of MEN 2A with variable nonendocrine manifestations have been recognized: MEN 2A with cutaneous lichen amyloidosis, which is characterized by pruritic lesions composed of subepidermal keratin deposits over the scapular region (Gagel et al., 1989); and MEN 2A with partial or extensive Hirschsprung syndrome, which represents another distinct clinical syndrome (Borrego et al., 1998). Patients with this disease exhibit evidence of both RET hyperfunction and hypofunction in a tissue--specific fashion (Eng and Mulligan, 1997).

Skeletal findings like elongated facies with a long, relatively thin nose, proliferation of corneal nerves, mucosal neuromas of the lips and tongue, and gastrointestinal ganglioneuromas have been found in virtually all MEN 2B patients (Williams and Pollock, 1966; Fryns and Chrzanowska, 1988; Morrison and Nevin, 1996). In addition, these patients frequently have aggressive tumors, both MTC and pheochromocytoma. These manifestations all appear to stem from abnormal proliferation of neural crest elements during fetal and postnatal life and be orchestrated by a hyperfunctioning RET tyrosine kinase receptor (Eng, 1996; Eng et al., 1996).

It should be noted that inactivating mutations of RET and some of its ligands, including glial derived nerve growth factor (GDNF) (see below), have been found in familial cases of Hirschsprung disease, and its variants (Angrist et al., 1996; Eng and Mulligan, 1997; Decker et al., 1998; Ponder, 1999). Screening for *RET* gene mutations (and mutations of its ligands) for familial Hirschsprung disease is currently recommended.

Molecular genetic mechanisms of *RET* mutations

The *RET* protooncogene on chromosome 10q11.2 encodes a receptor tyrosine kinase module. The RET protein consists of three functional domains: a large intracellular tyrosine kinase domain (with two subdomains), a transmembrane region, and an extracellular domain that has four cadherin-like repeats and a cysteine rich region. The ligands known to interact with RET include glial-derived neurotrophic factor (GDNF), neurturin (NRTN), artemin (ARTN), persephin (PSPN). There is also a family of membrane bound co-receptors termed GFRa-1, -2, -3, and -4. To activate RET, the ligand first binds with the requisite co-receptor, which then interacts on the cell membrane with the RET protein to cause receptor dimerization and initiation of intracellular signaling through the tyrosine kinase domains.

Oncogenic RET proteins activate a complex network of signal transduction pathways that contributes to cellular transformation. Binding of the ligand GFRa

Table 1. *RET* mutation[a, b] and associated clinical conditions.

EXON/MUTATION	PHENOTYPE
5	
G321R	FMTC
8	
G533C	FMTC, MEN2A
dupl E529-C531	FMTC
10	
dupl G592-G607	s. MTC
K603N	FMTC, s. MTC
C609G/R/S/Y/W	MEN 2A, FMTC, s. MTC, s. pheo
611G/F/R/S/Y	MEN 2A/ FMTC, s. MTC
del F612-C620	Sporadic MTC
C618G/F/R/S/Y	MEN 2A/ FMTC, s. MTC, s. pheo
C620G/F/R/S/W/Y	MEN 2A/ FMTC, s. MTC, s. pheo
11	
C630F/S/Y/R	MEN2A, s. pheo
D631G	s. MTC
C634F/G/R/S/W/Y/T/A	MEN 2A, FMTC, s. MTC, s. pheo
del D631-L-633	s. MTC
del E632-C634	s. MTC
del C630-D631	s. MTC
del E632-L633	s. MTC, s. pheo
E632S (del L-633-R635) T636S	s. MTC
del E632-A640 /ins VRP	s. MTC
E632D/ L633V /C634R	MEN2A
A639G/ A641R	s. MTC
12	
G748C	s. MTC
13	
P766S	s. MTC
E768D	FMTC, s. MTC
N777S	Low penetrance, non aggressive FMTC
L790F	MEN 2A, FMTC, s. pheo
Y791F	FMTC
13/14	
V778I/ V804M	FMTC
14	
V804L/M	FMTC
V804M/E805K	MEN2B
V804M/ Y806C	MEN2B
V804M/ R844L	FMTC
R833C	s. MTC
14/15	
V804/ S904C	MEN2B
15	
A876V	FMTC
A883F/T	MEN2B, FMTC, s. MTC
E884K	s. MTC
R886W	FMTC, MEN2
S891A	FMTC, MEN2
del D898-E902	s. MTC
16	
R912P	FMTC
M918T	MEN2B, s. MTC, s. pheo
S922F/P	s. MTC
D925H	s. pheo
T930M	s. MTC

MEN2: multiple endocrine neoplasia type 2; FMTC: familiar medullary thyroid carcinoma; s. MTC: sporadic medullary thyroid carcinoma; s. pheo: sporadic pheochromocytoma; del: deletion; ins: insertion; dupl: duplication; a: COSMIC Database -Catalogue of Somatic Mutations in Cancer; b: (Bethanis S et al., 2007; Cranston AN et al., 2006; D'Aloiso L et al., 2006; Dvorakova S et al., 2005; Prazeres HJ et al., 2006).

complex to RET triggers its homodimerization, phosphorylation of tyrosine residues and subsequent intracellular signalling; subsequently, RET activation leads to increased proliferation through a complex-network of second messengers, and the molecular partners and/or targets include Jun N-terminal kinase (JNK); mammalian target of rapamycin (m-TOR); phosphatidyl- inositol 3 kinase (PI3K), son of sevenless (SOS); vascular endothelial growth factor (VEGF); growth actor receptor bound protein 2 (GRB2), hypoxia-inducible actor 1a (HIF1a), extracellular signal-regulated kinase (ERK), protein kinase C (PKC), puruvate deydrogenase kinase (PDK), phospholipase Cγ (PLCγ). (Fig. 1)

Dominant activating mutations in the *RET* proto-oncogene have been identified as the main cause for the development of MTC. (Table 1). Germline activating mutations in the *RET* proto-oncogene are localized mostly in exons 10, 11, and 13 to 16. Missense mutations at one of six cysteine codons (609, 611, 618, 620, 630 at exon 10 and 634 at exon 11) which result in the substitution of any one of these extracytoplasmic cysteines by a different amino acid, are responsible for the majority of cases of MEN 2A (93-98%) (Blaugrund et al., 1994; Zedenius et al., 1994). Isolated FMTC was also caused by mutations in the first tyrosine kinase domain of the receptor (Eng, 1996; Eng et al., 1996). The cysteine-to-arginine change in codon 634 of the RET protein appears to be the most common mutation in the MEN 2 syndromes and is also present in the few kindreds with MEN 2A that have pheochromocytoma and hyperparathyroidism without other clinical manifestations.

In more than 95% of cases, MEN 2B is associated

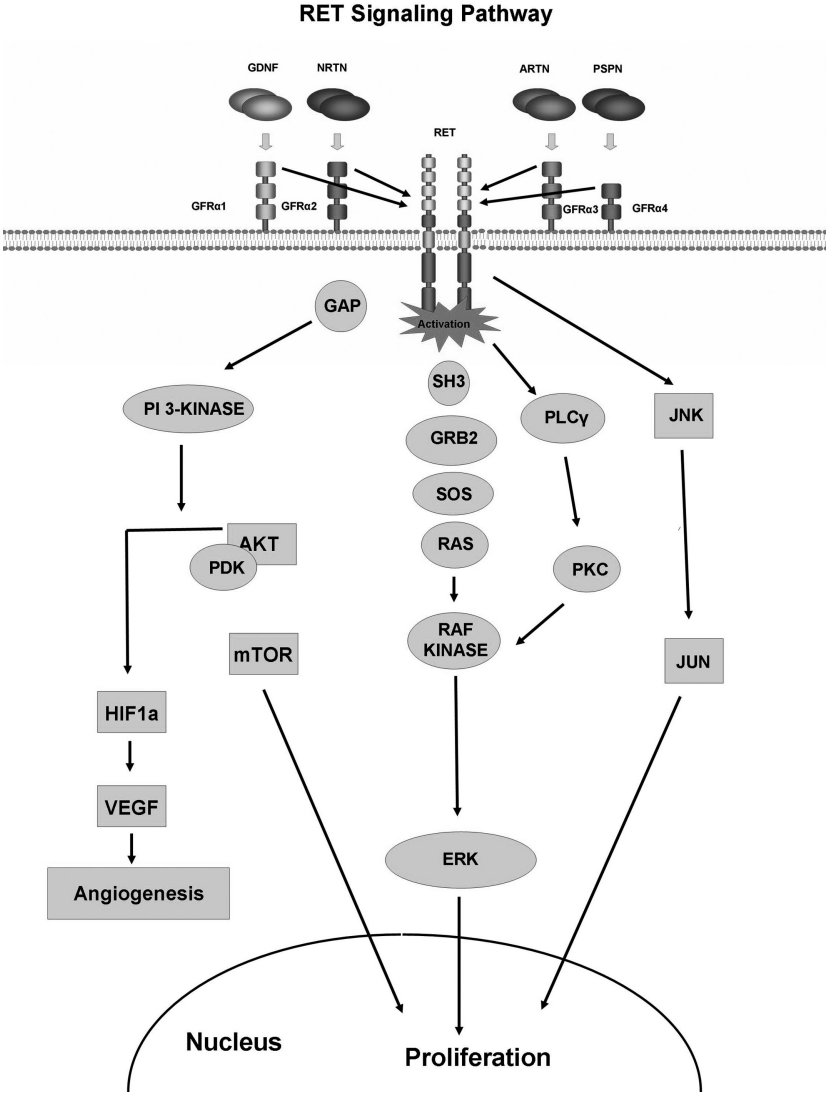

Fig. 1. Molecular biology of RET signalling: homodimeric GDNF-family ligands (GFLs) activate the transmembrane RET tyrosine kinase by binding to different GFR receptors; binding of the ligand GFRa complex to RET triggers its homodimerization, phosphorylation of tyrosine residues and subsequent intracellular signalling. RET activation leads to increased proliferation through a complex-network of second and third messengers that is developmentally-dependent and tissue-specific. This figure is also a composite of data from references cited in the text. NRTN: neurturin; ARTN: artemin; PSPN: persephin; JNK: Jun N-terminal kinase; m-TOR: mammalian target of rapamycin; PI3K: phosphatidylinositol 3 kinase, SOS: son of sevenless; VEGF: vascular endothelial grow actor; GRB2: growth actor receptor bound protein 2; HIF1a: hypoxia inducible actor 1a; ERK: extracellular signal-regulated kinase; PKC: protein kinase C; PDK: Pyruvate dehydrogenase kinase; PLCγ: phospholipase Cγ.

with a point mutation in the methionine residue in exon 16 (codon 918) in the intracellular tyrosine kinase receptor domain of RET. Mutations can be *de novo* in about 50% of MEN 2B cases; therefore, many patients with MEN 2B lack a family history of the disease. These MEN 2B patients often experience a delay in diagnosis until signs of mucosal neuromas or palpable thyroid tumors are obvious. In MEN 2A with Hirschsprung disease, most kindreds have mutations in codons 609, 618, and 620 (Eng and Mulligan, 1997). A summary of the known mutations, their locations in the RET molecule, and the specific clinical syndromes associated with them are shown in Table 1.

Although inherited mutations of the RET proto-oncogene appear to be directly responsible for the development of MEN 2-associated tumors, the mechanism(s) explaining why only few of the tissues carrying and expressing the mutant gene develop tumors remain unknown (Huang et al., 2003). Additionally, with the exception of MTC, most tumors in MEN-2 do not develop at a very young age; patients rather develop these tumors as late as during the 7th decade. Activation of RET and subsequent tumor formation could occur by a "second hit" causing a dominant effect of the mutant RET allele, either through duplication of the mutant allele (in the form of trisomy 10) or through loss of the normal, wild-type allele of the RET gene (Mulligan et al., 1993; Huang et al., 2000, 2003).

RET genetic screening and prophylactic thyroidectomy for MTC

Over the past decades, there has been a continuing improvement in survival in MEN 2 families, largely attributable to the success of family screening programs, using provocative biochemical tests and, more recently, detection of *RET* gene mutations. For several decades biochemical testing has been the principal means for detecting MTC. Since MTCs are derived from the calcitonin-producing C cells of the thyroid, an increased C-cell mass is reflected by an increased level of circulating calcitonin in the blood. Calcitonin is a good tumor marker, particularly when it is measured following its release from C cells by pharmacologic stimulation with calcium and pentagastrin. Before *RET* mutation analysis was available, the pentagastrin stimulation test was essential for early identification of affected individuals in families at risk for MEN 2A or FMTC albeit relatively inaccurate RET gene analysis has superseded older methods because of its lower cost, high sensitivity and specificity (Eng, 1996; Eng et al., 1996).

RET mutations have been grouped in 3 risk levels, regarding the predisposition to MTC. Level 1 mutations (in *RET* codons 609, 768, 790, 791, 804, and 891) lead to a late and slow-growing MTC; level 2 mutations (in *RET* codons 611, 618, 620, and 634) are mostly associated with CCH and/or MTC before the age of 5 years; and level 3 mutations (in RET codons 883 and 918), for which thyroidectomy is recommended at an early age (Eng, 1996; Eng et al., 1996; Evans et al., 2007). RET genetic testing is widely available (www.Genetests.org). Currently is common practice in most labs to sequence exons 10, 11, and 13 to 16 for MTC patients regardless of family history or clinical presentation, because only rare mutations are missed that

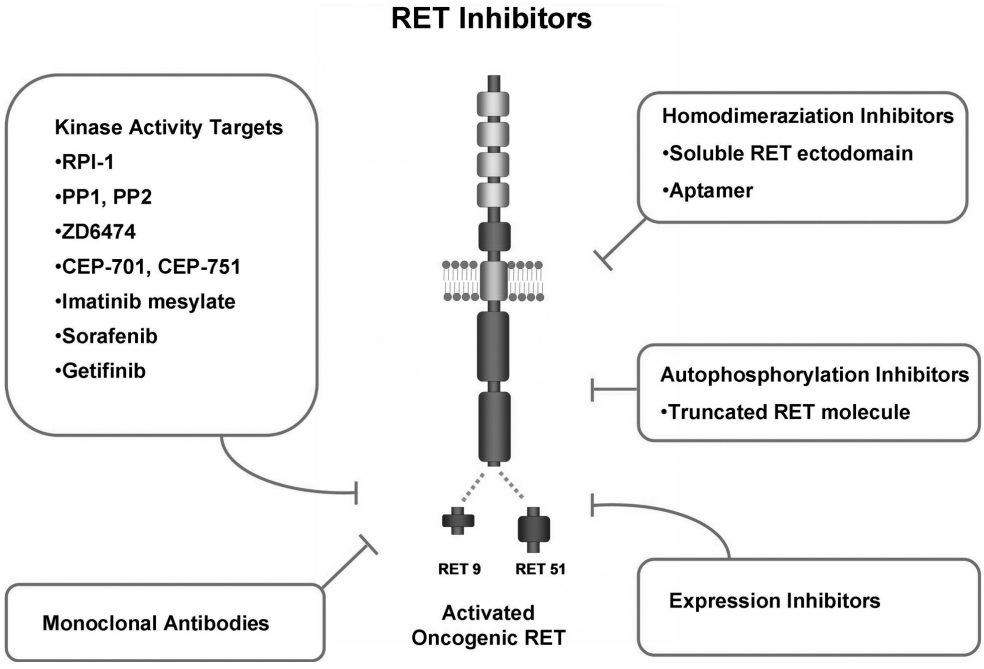

Fig. 2. A number of novel approaches are used to treat MTC and/or related neoplasms in which RET plays a role: kinase activity, homodimerization, expression and autophosphorylation inhibitors are directed various the various domains and/or function of the protein. In addition, monoclonal antibodies are in development. PP1: pyrazolopyrimidine 1; PP2: pyrazolopyrimidine 2; RPI-1: a 2-indolinone RET tyrosine inhibitor.

way. Targeted exon analysis (in exons 15 and 16 of the RET oncogene)is recommended for MEN 2B patients.

The most important consideration with regard to children known to have inherited a mutated *RET* allele is the timing of the thyroidectomy. Dr Skinner et al. showed that there was a lower incidence of persistent or recurrent disease in children who underwent total thyroidectomy before eight years of age and who had no metastases to cervical lymph nodes (Michael et al., 2005). The initial recommendation for prophylactic thyroidectomy before the age of 5 years for all carriers of *RET* mutations predisposing to MTC, has now been replaced by type-of-mutation-dependent guidelines for both the extent and age of surgery (Evans et al., 2007).

Advances in MTC prognosis and RET-kinase inhibitors in the treatment of MTC

MTC is the most common cause of death in patients with MEN 2a, MEN 2b, or FMTC, and the tumor is relatively unresponsive to conventional doses of radiation therapy and to standard or novel chemotherapeutic regimens (Gharib et al., 1992; Kebebew et al., 2000; Clayman and el-Baradie, 2003; Gulben et al.,2006). Surgery is the only standard treatment. Patients with MTC can be cured only by thyroidectomy, but only when it is performed at a time when the tumor is confined to the thyroid gland. Early recognition through genetic screening and detection of one of the characteristic mutations (see above) followed by prophylactic thyroidectomy has become the standard of care (Evans et al., 2007). In large series, patients who were detected by screening and who subsequently had prophylactic total thyroidectomy had 5 and 10 years survival rates approaching 100%. However, genetic screening is only applicable to those with a family history of MEN or FMTC. There is no treatment that has been shown to be effective for recurrent or persistent MTC. In addition, over half of the patients with MEN 2A and 2B present with advanced or even metastatic disease, and a large majority of the patients with MEN 2B represent new mutations, that could not have been detected by genetic screening. MTC metastasizes to local and regional lymph nodes, lung, bone and liver (Clayman and el-Baradie, 2003; Gulben et al., 2006). The survival in patients with advanced or residual local tumor or metastatic disease can be prolonged and even patients with metastatic MTC can remain asymptomatic and live for many years: on average, survival in patients who are not cured by thyroidectomy and have persistent local or metastatic disease is 80% and 70% at 5 and 10 years, and 5-year survival rate for patients with stage 4 disease exceeds 50%.

RET-kinase inhibitors have been developed recently for the treatment of MTC and are currently at various phases of pre- and clinical trials (Carlomagno et al., 2002a,b). Recently, a group initiated a phase II clinical trial evaluating the efficacy of oral ZD6474 (Zactima®) in patients with locally advanced or metastatic MTC: of the 20 patients accrued to date, objective remissions have been noted in about 30% (Lakhani et al., 2007). Other inhibitors of RET activity are under development targeting various aspects of the molecular biology of its signaling pathway (Figure 2) (Carlomagno et al., 2002a,b, 2003, 2006; Cohen et al., 2002; Cerchia et al., 2003, 2005; Strock et al., 2003; Cuccuru et al., 2004; Ezzat et al., 2005; Vidal et al., 2005; Strock et al., 2006).

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