UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS MÉDICAS: ENDOCRINOLOGIA

INFLUÊNCIA DAS VARIANTES GENÉTICAS DO PROTO-ONCOGENE *RET* NA APRESENTAÇÃO CLÍNICA DA NEOPLASIA ENDÓCRINA MÚLTIPLA TIPO 2

DÉBORA RODRIGUES SIQUEIRA

Porto Alegre, setembro de 2012.

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TESE DE DOUTORADO

DÉBORA RODRIGUES SIQUEIRA

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Médicas: Endocrinologia da Universidade Federal do Rio Grande do Sul como requisito parcial para obtenção do título de Doutor em endocrinologia.

Orientadora: Prof[a]. Dr[a]. Ana Luiza Maia

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Esta Tese de Doutorado segue o formato proposto pelo Programa de Pós-Graduação em Ciências Médicas: Endocrinologia, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, sendo apresentada na forma de 4 manuscritos sobre o tema da Tese:

- Artigo de revisão (1) (escrito em co-autoria): Molecular Basis of Medullary Thyroid Carcinoma: The Role of *RET* Polymorphisms; publicado no International Journal of Molecular Sciences 2012; 13:221-239.

- Artigo original (1): The *RET* polymorphic allele S836S is associated with early metastatic disease in patients with hereditary or sporadic medullary thyroid carcinoma; publicado no Endocrine-Related Cancer 2010; 17: 953–963.

- Artigo de revisão (2): Molecular Signaling Pathways and Targeted Therapy in Medullary Thyroid Carcinoma; encaminhado para publicação em periódico científico de circulação internacional.

Além dos artigos já citados, ao longo do período do doutorado foram desenvolvidos os seguintes manuscritos relacionados ao tema Neoplasia Endócrina Múltipla / Carcinoma Medular de Tireóide:

- Pancreatitis as the first manifestation of multiple endocrine neoplasia type 2A. Dora JM, **Siqueira DR**, Meyer EL, Puñales MK, Maia AL. Arquivos Brasileiros de Endocrinologia e Metabologia 2008; 52 (8):1332-6.

- Identification of occult metastases of medullary thyroid carcinoma by calcitonin measurement in washout fluid from fine needle aspiration of cervical lymph node. **Siqueira DR**, Rocha AP, Puñales MK, Maia AL. Arquivos Brasileiros de Endocrinologia e Metabologia 2009; 53 (4):479-81.

- Increased expression of vascular endothelial growth factor and its receptors, VEGFR-1 and VEGFR-2, in medullary thyroid carcinoma. Capp C, Wajner SM, **Siqueira DR**, Brasil BA, Meurer L, Maia AL. Thyroid 2010; 20 (8):863-71.

- Additive effect of RET polymorphisms on sporadic medullary thyroid carcinoma susceptibility and tumor aggressiveness. Ceolin L, **Siqueira DR**, Ferreira CV, Romitti M, Maia SC, Leiria L, Crispim D, Ashton-Prolla P, Maia AL. European Journal of Endocrinology 2012; 166 (5):847-54.

- The role of angiogenesis markers in pheochromocytoma. **Siqueira DR**, Romitti M, Ceolin L, Ferreira CV, Capp C, Brasil BA, Meurer L, Maia AL. Manuscripto em preparação.

LISTA DE ABREVIATURAS E SIGLAS

ABI - ankle-brachial index

ANRIL - antisense non-coding RNA

APC - Adenomatous polyposis coli

ATA – American Thyroid Association

BDNF - Brain-derived neurotrophic factor

BRAP - BRCA-1 associated protein

CCH - C Cell hyperplasia

CDK - Cyclin-dependent kinase

CEA - Carcinoembryonic antigen

CLA – Cutaneous lichen amyloidosis

CML - Chronic myelogenous leukemia

CT – Computed tomography

DNA - Deoxyribonucleic acid

c- kit - Stem cell factor receptor

EGF - Epidermal growth factor

EGFR - Epidermal growth factor receptor

ERK - Extracellular-signal-regulated kinase

FDA - Food and Drug Administration

FMTC – Familial medullary thyroid carcinoma

GDNF - Glial cell line-derived neurotrophic factor

GDP - Guanosine diphosphate

GFL – GNDF family of ligands

GFR α – GNDF family α receptor

GNEF - Guanine nucleotide-exchange factor

GTP - Guanosine triphosphate

HGF - Hepatocyte growth factor

HIRS - Hirschsprung's disease

HPT- Hyperparathyroidism

IKB - Inhibitors of NF-κB

IKK - IKB kinase complex

JAK - Janus kinase

JNK - c-Jun N-terminal kinase

LIF - Leukemia inhibitory factor

MAPK - Mitogen-activated protein kinase

MEN 2A- Multiple endocrine neoplasia type 2 A

MEN 2B- Multiple endocrine neoplasia type 2 B

MET -Hepatocyte growth factor

mRNA- Messenger ribonucleic acid

mTOR - Mammalian target of rapamycin

MTC – Medullary thyroid carcinoma

NF 1 – Neurofibromin 1

NFAT transcription factor – Nuclear factor of activated T-cells

NF-kB - Nuclear factor kappa B

PCLγ - Phospholipase C gamma

PCR - Polymerase chain reaction

PDGFR - Platelet-derived growth factor receptor

PDPK1 – 3-phosphoinositide-dependent protein kinase-1

PHEO – Pheochromocytoma

PIGF - Placental growth factor

PI3K - Phosphatidylinositol 3-kinase

PIK3CA - Catalytic subunit p110α of PI3K

PIP3 - Phosphatidylinositol 3,4,5 phosphate

PN1 - Lymph node metastasis

PM1 - Distant metastasis

PTEN - Phosphatase and tensin homolog

PTH – Parathyroid hormone

Rb – Retinoblastoma

RECIST - Response Evaluation Criteria in Solid Tumors

REST - RE1-silencing transcription factor

RET – RE arrangement during transfection

RFLP - Restriction fragment length polymorphism

RR - Relative risk

RTK - Receptor tyrosine kinase

SD - Stable disease

SNP – Single nucleotide polymorphism

sPHEO – Sporadic pheochromocytoma

STAT - signal transducer and activator of transcription proteins

TCF/LEF - T-cell factor/lymphoid enhancer factor

TGF α - Transforming growth factor α

TK - Tyrosine kinase

TKI - Inhibitors of tyrosine kinase receptors

TNM - Tumor/Node/Metastasis system

TP53 - Tumor protein p53

TPS1 - Thrombospondin-1

VEGF-A- Vascular endothelial growth factor A

VEGFR - Vascular endothelial growth factor receptor

VHL - von Hippel-Lindau disease

WT – Wild type

SUMÁRIO

RESUMO

O carcinoma medular de tireóide (CMT) é uma neoplasia das células C ou parafoliculares da tireóide, correspondendo a 5–8% dos tumores malignos da glândula. O CMT apresenta-se como um tumor esporádico (75-80%) ou na forma hereditária (20-25%). Na forma familiar é um dos componentes de uma síndrome genética de herança autossômica dominante, apresentando-se isoladamente, como carcinoma medular de tireóide familiar (CMTF) ou como um dos componentes da neoplasia endócrina múltipla (NEM) 2A ou 2B. A síndrome genética NEM 2A caracteriza-se pela presença de CMT (95%), feocromocitoma (30 – 50%) e hiperparatireoidismo (10-20%), enquanto que pacientes com NEM 2B podem aprensentar CMT (90%), feocromocitoma (45%), ganglioneuromatose (100%) e hábito marfanóide (65%).

O proto-oncogene *RET*, gene responsável pela NEM 2, codifica um receptor tirosino-quinase expresso nas células derivadas da crista neural. Mutações germinativas no *RET* são descritas principalmente nos exons 10, 11 e 16. Diversos estudos demonstraram a associação entre mutações específicas (genótipo) e idade no diagnóstico ou agressividade do CMT hereditário (fenótipo). No entanto, pacientes portadores da mesma mutação no proto-ongene *RET* podem apresentam quadros clínicos diferentes. Por esta razão, os polimorfismos do *RET* têm sido investigados como possíveis modificadores do fenótipo clínico em pacientes com NEM 2. Porém, os dados existentes na literatura sobre o papel dos polimorfismos do *RET* ainda são controversos.

O objetivo do nosso estudo foi avaliar o papel das variantes genéticas do *RET* na apresentação clínica do CMT *(Siqueira DR et al, Endocr Relat Cancer 2010; 17:953-963*). Dentro deste contexto, realizamos uma revisão do conhecimento atual sobre a associação dos polimorfismos do *RET* com o risco de desenvolver ou modificar a evolução do CMT (*Ceolin L et al, Int J Mol Sci 2012; 13:221-239*).

A melhor compreensão dos diferentes mecanismos moleculares envolvidos na patogênese tumoral permitiu o desenvolvimento de novos tratamentos, direcionados principalmente aos pacientes com doença metastática. Dentre as diversas classes de novas drogas, destacam-se os inibidores tirosina quinase; essas drogas inibem a ação de vários receptores, entre eles o RET. O crescente número de estudos publicados avaliando o efeito de inibidores tirosina-quinase no manejo de pacientes com CMT metastático determinou um estudo da literatura sobre este tema (*artigo submetido à publicação*).

Parte I

Molecular Basis of Medullary Thyroid Carcinoma: The Role of *RET* Polymorphisms

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(Qualis B1)

Molecular Basis of Medullary Thyroid Carcinoma: The Role of *Ret* Polymorphisms

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Abstract: Medullary thyroid carcinoma is a rare malignant tumor originating in parafollicular C cells. It accounts for 5 to 8% of all thyroid cancers. MTC develops in either sporadic (75%) or hereditary form (25%). Genetic and molecular studies have demonstrated the involvement of the *RET* proto-oncogene in hereditary MTC and, less often, in its sporadic form. Although a strong genotype-phenotype correlation has been described, wide clinical heterogeneity is observed among families with the same *RET* mutation or even in carriers of the same kindred. In recent years, several single nucleotide polymorphisms of the *RET* gene have been described in the general population as well as in patients with MTC. Some studies have reported associations between the presence of polymorphisms and development or progression of MTC. Nonetheless, other studies failed to demonstrate any effect of the *RET* variants. Differences in the genetic background of distinct populations or methodological approaches have been suggested as potential reasons for the conflicting results. Here, we review current knowledge concerning the molecular pathogenesis of sporadic and hereditary MTC. Particularly, we analyze the role of *RET* polymorphisms in the clinical presentation and prognosis of MTC based on the current literature.

Keywords: medullary thyroid carcinoma; *RET* polymorphisms; prognosis

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1. Molecular Basis of Medullary Thyroid Carcinoma

Medullary thyroid carcinoma (MTC) is a rare malignant tumor originating in parafollicular C cells of the thyroid first described by Hazard et al [1]. MTC accounts for 5 to 8% of all thyroid gland tumors and its main secretory product is calcitonin. MTC may occur sporadically, in approximately 75% of cases, or as part of the inherited cancer syndrome known as multiple endocrine neoplasia type 2 (MEN 2) [2-4]. The reported 10-year mortality rate for patients with MTC varies from 13.5 to 38% [5-7].

The hereditary form of MTC is associated with germline mutations in the *RET* (*REarranged during Transfection*) proto-oncogene, and presents as an autosomal dominant disease with a high penetrance and variable phenotype. *RET* point mutations are described mainly in exons 10, 11 and 16. However, less frequent mutations also occur in exons 5, 8, 13, 14 and 15 [8-13]. Hereditary MTC, also referred to as MEN 2, may be classified into three clinically distinct forms: multiple endocrine neoplasia type 2A (MEN 2A), type 2B (MEN 2B) and familial medullary thyroid carcinoma (FMTC) [11, 12].

The molecular mechanisms involved in the sporadic MTC have not yet been clarified. About 50 – 80% of the cases present a somatic *RET* mutation M918T (Met/ATG → Thr/ACG, exon 16) [14-17]. However, the mutation does not appear to be uniform among the various cell subpopulations in the tumor or in the metastases, suggesting that sporadic MTC might be of polyclonal origin, or that the mutations in the *RET* proto-oncogene are not initial events in MTC tumorigenesis [14, 16].

This review aims at presenting an updated picture of the current knowledge on the molecular pathogenesis of sporadic and hereditary MTC. Particularly, we critically analyze the role of *RET* polymorphisms in the clinical presentation and prognosis of MTC.

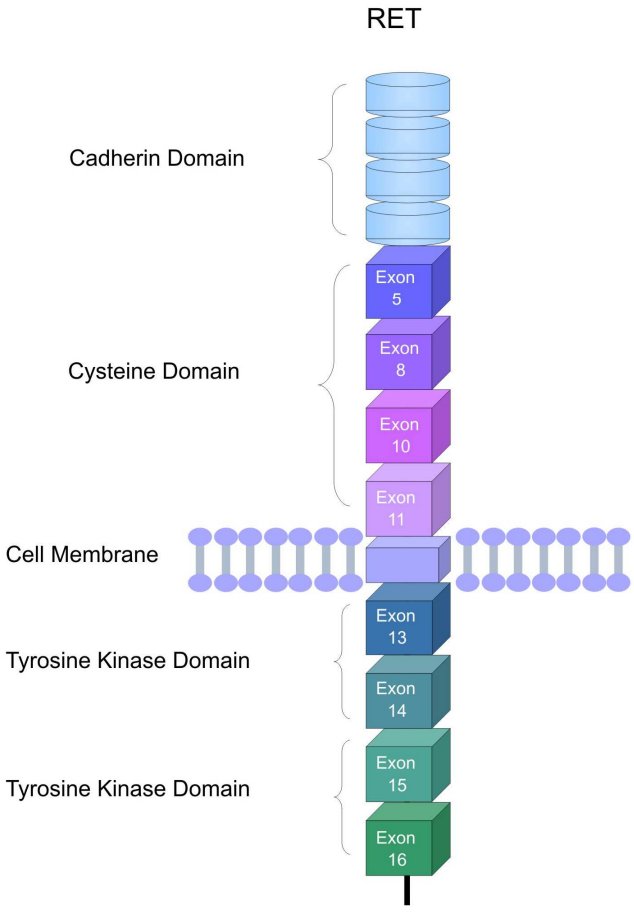
2. The *Ret* Proto-Oncogene

Genetic and molecular studies have shown the contribution of the *RET* proto-oncogene in hereditary MTC and, less often, in its sporadic form. The *RET* gene was identified in 1985 by Takahashi et al. during a classical experiment of NIH 3T3 cell transfection with the high molecular weight DNA of human T-cell lymphoma, hence the naming of the gene as *RET* (*REarranged during Transfection*) [18]. Later, studies determined the *RET* location in chromosome 10 and related it to the genesis of MEN 2A, MEN 2B and FMTC [19, 20]. In 1993, for the first time, point mutations in the *RET* gene were described in patients with MEN 2A and FMTC [9, 13] and in the subsequent year, a specific *RET* mutation (M918T) was associated with MEN 2B and sporadic MTC [21].

The *RET* gene encodes a receptor tyrosine-kinase, expressed in the cells derived from the neural crest: thyroid parathyroid cells (C cells), chromafin cells of the adrenal medulla and enteric autonomic plexus. Since it is a membrane receptor, the RET protein is constituted by three domains: an extracellular domain, a transmembrane domain and an intracellular portion containing two tyrosine-kinase domains (Figure 1). The extracellular domain includes regions homologous to the cadherin family of cell adhesion molecules and a large region rich in cysteine residues that performs the transduction of extracellular signals of proliferation, growth, differentiation, migration, survival and cell apoptosis. The intracellular domain is divided into 2 tyrosine-kinase subdomains (TK1 and

TK2), separated by 28 aminoacids. These subdomains contain the tyrosine residues that are phosphorylated during receptor activation, and are involved in the activation of the signaling intracellular pathways. *RET* is subject to alternative splicing of the 3' region generating three protein isoforms that contain 9 (*RET*9), 43 (*RET*43) and 51 (*RET*51) amino acids in the carboxy-terminal tail downstream from glycine 1063. *RET*9 and *RET*51, consisting of 1072 and 1114 amino acids, respectively, are the main isoforms in vivo [22, 23].

Figure 1. Schematic representation of the RET receptor. The extracellular region comprises the cadherin and cysteine rich domain. A single transmembrane region spans the cell membrane. Two tyrosine kinase domains (TK1 and TK2) are located in the intracellular region. The corresponding exons coding for the cysteine and thyrosine kinase domains are indicated.

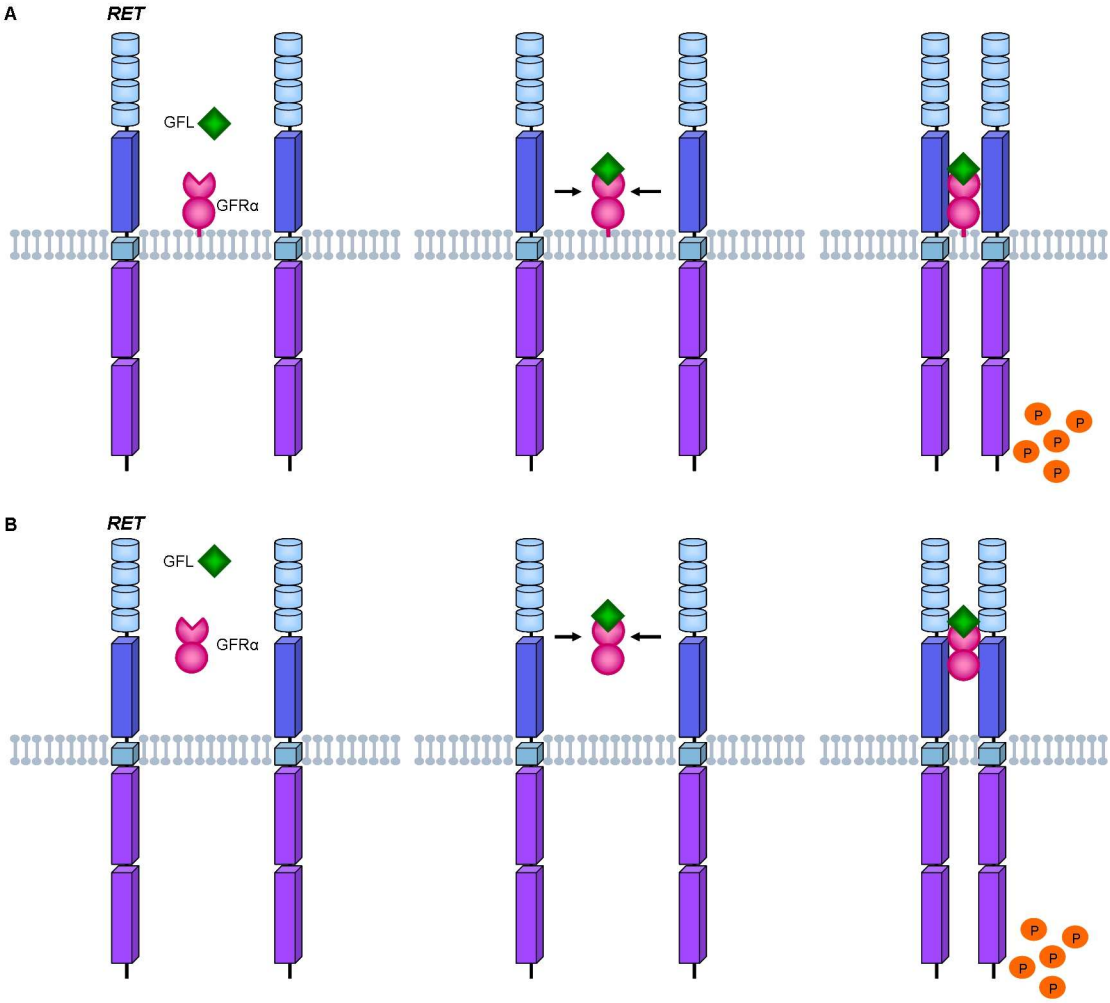


3. *Ret* Protein Activation

The RET receptor tyrosine kinase is activated through a complex formed by the glial cell line-derived neurotrophic factor (GDNF) family of ligands and co-receptors. Under normal conditions, RET activation depends on the interaction of GFRαs (GDNF Family α Receptor) co-receptors and their respective ligands GFLs (GDNF Family of Ligands). The GFRα-ligand complex, together with the extracellular portion of RET, promotes autophosphorylation of the intracellular tyrosine residues [24, 25].

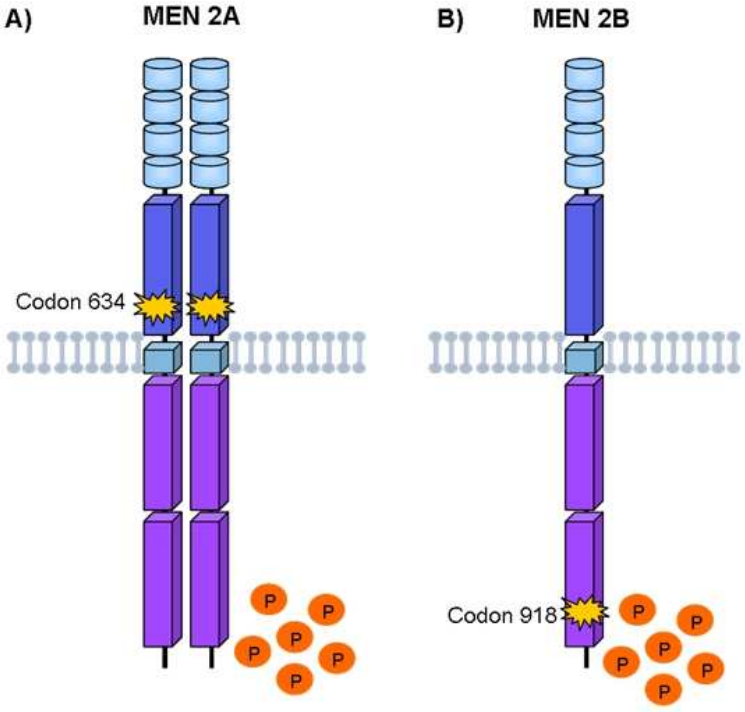
The RET co-receptors are usually bound to the plasma membrane, but GFRs also occur in a soluble form, and can then activate RET in two distinct forms: *cis* or *trans* (Figure 2). The *cis* model for the RET activation hypothesis occurs when the GFL ligand binds to the GFR*α* co-receptor anchored on a lipid platform and later this complex promotes the approach of two RET molecules through the lipid platform, allowing the phosphorylation of the intracellular tyrosine residues. On the other hand, the *trans* model activation suggests that the GFL may also bind to the soluble form of GFRα, stimulating the dimerization of RET outside the lipid platform, thus allowing its activation. Once activated, RET initiates the different intracellular pathways involving the regulation of processes such as differentiation, survival, proliferation, migration and cell chemotaxis [24, 26].

Figure 2. Mechanisms of ligand-mediated RET activation. (**A**) In the *cis* model RET activation: the GDNF family of ligands (GFL) binds to membrane glycosylphosphatidylinositol-anchored GDNF-family coreceptors (GFRα). The activation leads to dimerization of RET and consequently activation of the intracellular signaling pathways. (**B**) In the *trans* model RET activation: the ligand binds to the soluble form of its coreceptor (GFRα) and the ligand-GFRα complex brings together two inactive RET monomers. Ligand-induced activation induces dimerization and tyrosine phosphorylation of the RET receptor with downstream activation of several signal transduction pathways.



The molecular mechanism by which *RET* mutations trigger the neoplastic process was determined by elegant *in vitro* studies performed by Santoro *et al.* [27]. Briefly, under normal conditions, RET is only activated in the presence of GFRα/GFL complex, which on binding to the RET receptor promotes its dimerization and auto-phosphorylation of the intracellular signaling pathways. The presence of mutation in the extracellular domain, as found in MEN 2A, leads to the dimerization of RET even in the absence of the ligand, with consequent constitutive activation of the intracellular signaling pathways (Figure 3A). Mutations in the intracellular tyrosine-kinase domain, as found in MEN 2B, alter RET substrate specificity due to structural changes in this domain. Consequently, the mutated RET no longer needs dimerization to become active (Figure 3B) [28, 29]. The activation of the RET protein appears to be an initial step in the oncogenic pathway in the tissues where it is expressed. Molecular evidence of other chromosomal abnormalities, such as loss of heterozygosity, most often at 1p and 22q, suggest that additional cytogenetic events are probably involved [11, 30].

Figure 3. Characterization of RET oncogenic activation in MEN2 inherited cancer syndromes. (**A**) MEN 2A *RET* 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. (**B**) MEN 2B *RET* mutation activates tyrosines in the kinase domain and alters its substrate specificity leading to aberrant phosphorylation of substrates of RET receptor.



4. Hereditary Medullary Thyroid Carcinoma

Approximately 25% of MTC cases occur as part of the inherited cancer syndrome of MEN 2 [7, 31]. The MEN 2A subtype constitutes approximately 70%-80% of cases of MEN 2 and is characterized by the presence of MTC (95%), pheochromocytoma (30–50%) and hyperparathyroidism (HPT) (10–20%). The MEN 2B syndrome accounts for about 5% of the cases of MEN 2. The frequency of MTC is over 90%, pheochromocytoma (45%), ganglioneuromatosis (100%) and marfanoid habitus (65%) [11, 32]. This syndrome is characterized by a single phenotype, which includes diffuse ganglioneuromatosis of the tongue, lips, eyes and gastrointestinal tract, long fingers and extremities, hyperextension of the joints and epiphyseal abnormalities. MTC in the setting of MEN 2B develops earlier and has a more aggressive course, occurring at a younger age compared with MTC in other MEN 2 subtypes [6, 7]. The FMTC subtype constitutes approximately 10 to 20% of the cases of MEN 2 [11]. MTC is the only manifestation and thereby it is necessary to demonstrate the absence of a pheochromocytoma or hyperparathyroidism in two or more generations of the same family or the identification of related mutations to confirm that particular kindred have this syndrome. In these cases, the clinical presentation of MTC occurs later and the prognosis is more favorable (corresponding to older age at onset, often between 20 and 40 years) compared to the other forms of MTC [33].

4.4 Germline RET Mutations and Disease Phenotype

Several studies indicate a correlation among specific *RET* mutations (genotype) and age of onset, aggressiveness of MTC and the presence or absence of other endocrine neoplasms (phenotype) [11, 34-36]. Several independent mutations in the *RET* at exons 5, 8, 10, 11, 13, 14, 15 and 16, have been established as causative of MEN 2A, MEN 2B and FMTC [8-13].

The majority of families with MEN 2A (more than 90%) present point mutations in the *RET* proto-oncogene (*missense* type), involving codons located in the extracellular domain of the receptor: 609, 611, 618 and 620 (exon 10) and 634 (exon 11). The most frequent mutations are located in codon 634, occurring in more than 60% of all genetically identified MTC [11, 13, 32, 37]. Codon 634 mutations have been associated with the presence of pheochromocytoma and hyperparathyroidism [38], and rarely with CLA [39]. Nevertheless, there are a variety of phenotypic expressions in families with the same *RET* mutation [9, 11, 12, 35, 38]. Puñales et al observed that the genotype C634R (TGC/Cys → CGC/Arg, exon 11) presented significantly more distant metastases at diagnosis than groups C634W (Cys/TGC → Trp/TGG, exon 11) and C634Y (Cys/TGC → Tyr/TAC, exon 11), thus suggesting that a change of specific amino acids may modify the natural development of the disease [36]. A recent study evaluated the *RET* C634W-specific neoplastic risk and age-related penetrance profiles and found that penetrance is high for MTC (52% by age 30, 83% by age 50 and 98% by age 70) and pheochromocytoma (20% by age 30, 67% by age 50 and 92% by age 70) [40]. In contrast to well-defined risk profiles for carriers of the codon 634 mutations, consensual clinical guidelines for *RET* exon 10 mutation are still being defined. Risk profiles and penetrance estimations in MEN 2A caused by germline *RET* exon 10 mutations were recently analyzed by Frank-Raue et al (2011) in a large multicenter study that included 340 subjects from 103 families. The authors observed that mutations affect mainly the cysteine codons 609, 611, 618 and 620 and 50% penetrance was achieved by the age of 36 years for MTC, by 68 years for pheochromocytoma, and by 82 years for HPT [41]. These data may facilitate risk assessment and genetic counseling for MTC.

MEN 2B occurs, in approximately 95% of the cases, through a specific M918T mutation (exon 16), resulting in the structural change of the intracellular domain of the RET protein. In about 2%–3% of patients with MEN 2B, the genotype A883F (GCT → TTT, exon 15) can be found [42, 43]. In addition, a double mutation V804M/Y806C at codon 804 (Val/GTG → Met/ATG, exon 14) and 806 (Tyr/TAC → Cys/TGC) in the same allele was described in a patient with MEN 2B. Patients presenting with "atypical" MEN 2B harboring the germline double point mutation in codons 804 and 904 (V804M and S904C) were also reported [44, 45]. Mutations in codons 883 and 918 are associated with younger age of MTC onset and higher risk of metastases and disease-specific mortality [11, 31, 46].

In FMTC, germline mutations are distributed throughout the *RET* gene. Approximately 86-88% of FMTC families have mutations in one of the 5 cysteines in the extracellular domain of the *RET* gene in exons 10 (codons 609, 611, 618, 620) and exon 11 (codon 634) [12, 47]. Substitutions in the intracellular domain of *RET* in exon 13 (codon 768, 790, 791), in exon 14 (codon 804 and 844) and

in exon 15 (codon 891) are less frequent. Interestingly, the most frequent mutation in MEN 2A, C634R, has not been described in FMTC families [11, 47-50].

Based on genotype-phenotype correlation studies, the American Thyroid Association (ATA) developed recommendations for age of prophylactic thyroidectomy in asymptomatic *RET* mutation carriers. The different mutations are classified into four risk categories according to the aggressiveness of the disease (A<B<C<D). Children with mutations associated with MEN 2B phenotype (ATA level D risk) are at highest risk for early development of MTC and should have thyroidectomy as soon as possible, preferably within the first year of life. Patients with codon 634 mutations (ATA level C risk) are also at higher risk for development of MTC at early ages and the prophylactic total thyroidectomy should be carried out before 5 years of age. In patients with ATA level A and B *RET* mutations (codons 768, 790, 791, 804, 891 and 609, 611, 618, 620, 630 respectively), the risk for MTC is moderate and the prophylactic total thyroidectomy may be delayed beyond the age of 5 years if there is a less aggressive MTC family history, a normal basal stimulated serum calcitonin and normal neck ultrasound [51].

5. Sporadic Medullary Thyroid Carcinoma

Sporadic MTC generally presents as a unifocal tumor or a palpable cervical lymph node. Diagnosis tends to be late, generally in the fifth or sixth decade of life [52]. Lymph node metastases are detected in at least 50% of these patients, while distant metastases occur in ~ 20% of cases [53, 54]. A minority of patients with MTC present systemic manifestations which include diarrhea, flushing, or painful bone metastases [6].

5.1 Somatic RET Mutations and Disease Phenotype

In sporadic MTC, somatic mutation in exon 16 of the *RET* (M918T) has been identified in 50 – 80% of the patients [14-17]. Somatic mutations in codons 618, 603, 634, 768, 804 and 883 and partial deletion of the *RET* gene have been identified in few tumors [53, 54]. The presence of a somatic *RET* mutation correlates with a worse outcome for MTC patients, not only because of the higher probability of persistent disease, but also because of a lower survival rate in a long-term follow up [53, 54].

The somatic *RET* mutations (exons 10, 11 e 16) have also been described in other endocrine tumors. Mutations associated with MEN 2A (codon 634 and 631) and 2B (codon 918) phenotype are also found in about 15-20% of sporadic pheochromocytomas [55, 56].

6. Role of Polymorphisms in Medullary Thyroid Carcinoma

Since the identification of the *RET* proto-oncogene as the susceptibility gene for hereditary MTC, major advances have been observed in studies concerning the pathogenesis of MTC and associated neoplasias [9, 13]. However, certain aspects of the disease, such as the clinical

heterogeneity observed in individuals who have the same mutation, are not yet well understood [36, 57, 58]. As to sporadic MTC, the picture is slightly more obscure, since *RET* somatic mutations are not found in all cases [15, 21, 46, 59] and appear not to occur uniformly among the different subpopulations of cells in the tumor [14, 15, 60]. In recent years, several authors have investigated whether the presence of variant sequences or polymorphisms could be associated with susceptibility for the development or progression of MTC. These studies have described an increased prevalence of the *RET* polymorphisms G691S (exon 11, rs1799939), L769L (exon 13, rs1800861), S836S (exon 14, rs1800862), and S904S (exon 15, rs1800863) in individuals with hereditary or sporadic MTC when compared with the population [17, 57, 60-62]. Below, we will discuss the main aspects related to these polymorphisms and susceptibility to MTC development.

6.1 RET G691S and S904S Polymorphisms

The non synonymous variant G691S (Gly/GGT→Ser/AGT) has been associated with developing sporadic MTC in two larger studies [61, 63]. In an Italian population it was demonstrated that the frequency of G691S polymorphism was greater in patients with sporadic MTC compared to the controls (27.8% vs 18.9% P= 0.029). Moreover, the authors observed that G691S polymorphism presents a positive significant co-segregation with S904S (SerTCC→SerTCG) polymorphism [61]. Additionally, Cebrian et al (2005), have demonstrated a 1.5 to 2.5 -fold increase in the relative risk for the development of MTC in patients who presented polymorphisms in exons 11 (G691S), 15 (S904S) and 19 (STOP+388bp) [63]. These two studies postulated, through a functional assessment of *RET* transcription and splicing, that G691S could be the functional variant, but the results were inconclusive [61, 63]. Fugazzola, et al. 2008 also tested the functional activity of the *RET* G691S variant and show that the *RET*9-G691S protein was overrepresented when compared to *RET*9-WT. However, no transforming activity was observed [64].

Robledo et al, in 2003, also described a strong co-segregation between polymorphisms G691S and S904S, reporting a strong linkage disequilibrium between these polymorphisms. Additionally, it was also demonstrated that haplotype G691S/S904S, in homozygosis, was more prevalent in patients with MEN 2A compared to the control group, suggesting a role as a gene with low penetrance for this variant. Furthermore, the authors observed that this variant (G691S/S904S) could modify the age of onset of MTC patients [57]. However, these data were not replicated in a large sample of European population [65].

Although several studies have found an association between G691S/S904S polymorphisms and MTC, some authors did not observe a difference in the frequency of this variant between MTC patients and the general population [17, 66-68]. Wohllk *et al.* analyzed 50 Chilean patients with sporadic tumors and 50 controls of similar ethnic origins, and showed a similar frequency of the *RET* G691S/S904S variants for cases and controls [69]. More recently, these negative results were replicated in Polish, Brazilian and Indian populations [17, 67, 68].

6.2 RET L769L Polymorphism

In 2001, a study conducted by Wiench et al reported that patients with sporadic MTC and under the age of 30 years presented a higher frequency of the variant L769L (LeuCTT→LeuCTG) allele than those diagnosed between 31 – 45 years (36% vs. 15%, P=0.04), suggesting that this polymorphism was associated with younger age at diagnosis. However, the absence of a control group diminished the relevance of this observation [58]. Interestingly, Magalhães et al (2004) observed that a patient harboring a V804M mutation, classically associated with late-onset and lower aggressiveness MTC, associated with the L769L polymorphism presented clinically evident MTC at 32 years of age, in contrast to her asymptomatic mother, who had only the V804M mutation and had MTC diagnosed by fine-needle aspiration biopsy at 60 years of age. The authors suggest that polymorphism L769L of *RET* proto-oncogene may be related to younger age at the onset of disease [70].

An association between the presence of L769L polymorphism and F769Y mutation was reported in FMTC patients for Baumgartner-Parzer *et al*. In this study, the authors deduced from pedigree analyses that the F791Y mutation and L769L polymorphism are located on the same allele and speculated whether the presence of this polymorphism could predispose the respective allele for the occurrence of a F791Y *de novo* mutation or would modulate the disease phenotype [66].

More recently, the presence of polymorphism L769L in the *RET* gene was associated with predisposition to the development of sporadic MTC and also younger age at onset of MTC in carriers of the homozygous polymorphic variant L769L. The authors also demonstrated that this variant modifies the structure of mRNA and could lead to changes in kinase activity and/or specificity of the protein [68].

Conversely, other studies did not show an association between the L769L polymorphism and MTC [60, 61, 63, 69]. Berard et al. analyzed the presence of the L769L polymorphism in patients with sporadic MTC and controls, and found no difference in the distribution of these polymorphisms between the groups analyzed [71]. Accordingly, Siqueira et al. did not observe the influence of neutral *RET* L769L variants on clinical and oncological features in individuals with hereditary or sporadic MTC [17]. Recently, a study performed in Indian patients also failed to demonstrate a difference in the frequency of this allele in MTC patients and control group [67].

6.3 RET S836S Polymorphism

Gimm et al, in 1999, identified an association between the *RET* polymorphisms S836S (SerAGC→SerAGT) and sporadic MTC. The authors reported a higher frequency of the variant allele in the group with MTC compared with the control group (9.0 vs 3.7% P= 0.03) [60]. These findings were confirmed in a Spanish population [72]

A recent study investigated the influence of the neutral *RET* S836S variants on the clinical presentation of hereditary or sporadic MTC in a large cohort of Brazilian patients. The variant S836S was associated with the early onset of the disease and a higher risk for the development of lymph node and distant metastases (P=0.002 and P=0.001, respectively) in patients with hereditary or sporadic MTC [17].

Other association studies, however, have failed to show differences as to the presence of S836S polymorphisms between patients with sporadic MTC and controls [61, 63, 68, 69]. Wiench et al. in a Polish population and Berard et al. in French patients observed a similar frequency of the *RET* S836S variants for cases and controls [58, 71]. Similar data were found in other populations [61, 63, 68, 69]. Study performed in India did not observe significant differences in the frequency of this polymorphic allele in the patients and control group. Interestingly, the prevalence of the *RET* polymorphisms in the Indian population was significantly higher than those observed in Germans, Italians, French, Spanish and Hungarians (P>0.002) [67].

6.4 Other RET Variants

Besides the variants already mentioned, other polymorphisms have also been associated with MTC. A study showed higher frequency of intron 14 (IVS14–24; rs2472737) polymorphism in the group with elevated serum calcitonin concentrations (P = 0.016) and in patients with sporadic MTC (P < 0.001), when compared with the control group with normal calcitonin levels. However, further studies are necessary to characterize a potential role of this *RET* sequence variant in the development of sporadic MTC [66].

Recently, two other variants of *RET* were identified (IVS1–126 G > T; rs2565206) and (IVS8+82 A > G; rs3026750 and 85–86 insC; rs3482797), and associated with phenotypic variability in patients with mutation G533C. In this study, the authors found an association between variant IVS1–126G > T and age at diagnosis of MTC. On the other hand, variant [IVS8+82 A > G; InsC 85-86] was associated with the presence of lymph node metastases at the time of diagnosis. Analyses in silico suggest that this variant may induce abnormal splicing, postulating that variant [IVS8+82 A > G; 85-86 InsC] could interrupt and/or create an exonic splicing site, thus leading to the synthesis of an altered protein [73]. In another study, a polymorphism in exon 2 (GCG→GCA), which encodes an alanine (A45A), occurred at a lower frequency among the cases of MTC and, according to the authors, it could confer a protective allele against the development of MTC [63].

Taken together, these data point to a potential influence of *RET* variants in the development and progression of MTC. Tables 1 and 2 summarize the main findings of the studies on the role of *RET* polymorphisms in MTC.

Table 1. Role of the RET variants in hereditary medullary thyroid cancer.

RET variant	Author	Cases	Controls	P	Frequency (cases *vs.* controls)	Genotyping platform	Conclusion	Population
G691S (rs1799939)	Robledo (2003)	198	653	0.037	___	sequencing	Associated with the presence of MTC in younger individuals.	Spanish
	Lesueur (2006)	384	___	___	___	Taqman	N/A	European
	Tamanaha (2009)	77 [a]	100	0.048	0;4	RFLP	Underrepresented in G533C-arriers.	Brazilian
	Sharma [b] (2011)	51	50	NS	49;48	sequencing	N/A	Indian
L769L (rs1800861)	Sharma [b] (2011)	51	50	NS	45;58	sequencing	N/A	Indian
S836S (rs1800862)	Tamanaha (2009)	77 a	100	0.008	16.9; 4	RFLP	Over-represented in G533C-carriers.	Brazilian
	Siqueira (2010)	88	___	___	7.95; -	RFLP	Associated with early onset and increased risk for metastatic disease.	Brazilian
	Sharma b (2011)	51	50	NS	25;22	sequencing	N/A	Indian
S904S (rs1800863)	Lesueur (2006)	384	___	___	___	Taqman	N/A	European
	Sharma b (2011)	51	50	NS	25;22	sequencing	N/A	Indian
	Tamanaha (2009)	77 a	100	0.048	0;4	RFLP	Underrepresented in G533C-carriers.	Brazilian
IVS1-126 G>T (rs2565206)	Tamanaha (2009)	77 a	100	0.002	1.3;0	RFLP	Associated with younger age at diagnosis.	Brazilian
IVS8 +82 A>G; 85–86 insC (rs3026750)	Tamanaha (2009)	77 a	___	0·019	___	RFLP	Associated with lymph node metastases. Could induce abnormal splicing.	Brazilian

[a] Study performed in patients with *RET* G533C mutation; [b] The study included hereditary and sporadic MTC patients; N/A: no association was found.

Table 2. Role of the *RET* variants in sporadic medullary thyroid cancer.

RET variant	Author	Cases	Controls	P	Frequency (cases vs. controls)	Genotyping platform	Conclusion	Population
G691S/S904S (rs1799939)/ (rs1800863)	Elisei (2004)	106	106	0.029	27.8;18.8	RFLP	Higher frequency in MTC patients. Does not influence RET mRNA expression	European
	Cebrian [a] (2005)	120	528	0.004	27;18	TaqMan	Associated with higher risk for development of MTC. Does not affect the splicing of RET	British
	Wohllk (2005)	50	50	NS	25;25	sequencing	N/A	Chilean
L769L (rs1800861)	Wiench (2001)	116 [b]	-	0.04 [b]	36;15	sequencing	Associated with the presence of MTC in younger individuals.	Polish
	Sromek (2010)	217	420	0.039[c]	48.3;39.5 [c]	Sequencing	Associated with the presence of MTC in younger individuals (in homozygosis). Could influence RET mRNA structure.	Polish
	Berard (2004)	184	174	NS	22.3;25.9	sequencing	N/A	French
	Wohllk (2005)	50	50	NS	24;23	sequencing	N/A	Chilean
S836S (rs1800862)	Gimm (1999)	50	70	0.03	9;3.7	RFLP	More frequent in MTC patients.	German-American
	Ruiz (2001)	32	250	0.04	9.3;3.6	RFLP	Associated with higher risk for development of MTC.	Spanish
	Siqueira (2010)	81	80	0.01	10.5;3.2	RFLP	Associated with early onset and increased risk for metastatic disease.	Brazilian
	Berard (2004)	184	174	NS	6.5;5.2	sequencing	N/A	French

	Author	Cases	Controls	P	Frequency (cases vs. controls)	Genotyping platform	Conclusion	Population
	Wohllk (2005)	50	50	NS	6;1	sequencing	N/A	Chilean
***RET* variant**	**Author**	**Cases**	**Controls**	**P**	**Frequency (cases vs. controls)**	**Genotyping platform**	**Conclusion**	**Population**
S904S (rs1800863)	Wohllk (2005)	50	50	NS	27;28	sequencing	N/A	Chilean
	Cebrian (2005)	125	528	0.005	26.4;15.5	TaqMan	Associated with higher risk for development of MTC.	British
STOP+388pb G>A (rs3026782	Cebrian (2005)	123	522	0.005	26.4;15.5	TaqMan	Associated with higher risk for development of MTC.	British
A45A G>A (rs1800858)	Cebrian (2005)	126	525	0.04	21;27.9	TaqMan	Suggest protective effect	British

[a] Study did not confirm the previously described association between G691S and S904S; [b] The comparison was performed between patients aged below and above 30 years; [c] Frequency of heterozygous change L769L.N/A: no association was found.

6.5 Possible mechanisms of action for RET polymorphism in Medullary Thyroid Carcinoma

So far it is not known how polymorphisms exert their effects on the development or progression of MTC and the mechanistic explanation is still speculative. A quantitative study of *RET* mRNA levels in tumor tissues of individuals with MTC did not show a difference in the expression in patients with and without G691S/S904S polymorphism [61]. The S836S polymorphism failed to affect DNA–protein binding, transcript stability, or RNA splicing and editing [74]. Other hypothesis is that bases exchange in the DNA molecule could interrupt and/or create a splicing site, leading to the synthesis of an altered protein, or else, that the modified nucleotide is in a state of linkage disequilibrium with an as yet unknown functional variant [60, 73]. It has also been proposed a specific effect of G691S polymorphism on RET dimerization on MEN 2A patients harboring the 634 mutation [57]. Potential changes on mRNA structure due to the presence of RET polymorphisms have also been evaluated. The simplest prediction of mRNA structure is a prediction of thermodynamic stable structure, MFE (minimal free energy) structure. Bioinformatics analysis showed that differences in MFE between wild types and mutants are <5% in the case of polymorphisms S904S and S836S and mutations Y791F and C634R. No effect on MFE was visible also in the combination of C634R and L769L polymorphism. However, the difference was noticeable in the case of exon 13. The L769L variant reduces the energy of the wild type by 17% and the mutant Y791F by 7%, leading the authors to conclude that the L769L polymorphism reduces the MFE of small RET mRNA [68]. Finally, in silico analysis revealed that the IVS1–126G>T genetic variant creates a new binding site for NFAT transcription factor (nuclear factor of

activated T-cells) [75]. The NFAT family of proteins has been found to be involved in cell cycle regulation, cell differentiation, cell survival, angiogenesis, tumor cell invasion, and metastasis [76], which may explain the association of this variant with disease progression [73].

7. Conclusion

In summary, since the recognition of the RET proto-oncogene as the susceptibility gene for hereditary MTC several decades ago, advances have taken place in understanding pathogenesis of MTC and associated neoplasias. Nevertheless, certain aspects of the disease, such as the clinical heterogeneity seen in individuals harboring the same mutation have not yet been well understood. Polymorphisms in the RET gene are commonly associated with MTC and may partially explain the large clinical heterogeneity observed in MEN 2A patients. An entire set of data obtained from clinical studies indicates a potential role of RET polymorphisms in the development of sporadic MTC. However, in contrast, several others failed to demonstrate any association between these *RET* variants and MTC development or disease progression. Although differences in ethnic background or methodological flaws might be potential causes for the different results described, the mechanism underlying the positive associations is still lacking which stimulates further controversy. Since the contribution of a single variant to a disease is determined by the prevalence of the implicated allele and the magnitude of the association with the condition, the results summarized here might indicate the need for large multicenter studies to confirm or rule out a role of these variants as a cause or modifying agent in this rare disease.

References

1. Hazard, J. B.; Hawk, W. A.; Crile, G., Jr., Medullary (solid) carcinoma of the thyroid; a clinicopathologic entity. *J Clin Endocrinol Metab* **1959,** 19, (1), 152-61.

2. Gilliland, F. D.; Hunt, W. C.; Morris, D. M.; Key, C. R., Prognostic factors for thyroid carcinoma. A population-based study of 15,698 cases from the Surveillance, Epidemiology and End Results (SEER) program 1973-1991. *Cancer* **1997,** 79, (3), 564-73.

3. Raue, F.; Kotzerke, J.; Reinwein, D.; Schroder, S.; Roher, H. D.; Deckart, H.; Hofer, R.; Ritter, M.; Seif, F.; Buhr, H.; et al., Prognostic factors in medullary thyroid carcinoma: evaluation of 741 patients from the German Medullary Thyroid Carcinoma Register. *Clin Investig* **1993,** 71, (1), 7-12.

4. Saad, M. F.; Ordonez, N. G.; Rashid, R. K.; Guido, J. J.; Hill, C. S., Jr.; Hickey, R. C.; Samaan, N. A., Medullary carcinoma of the thyroid. A study of the clinical features and prognostic factors in 161 patients. *Medicine (Baltimore)* **1984,** 63, (6), 319-42.

5. Girelli, M. E.; Nacamulli, D.; Pelizzo, M. R.; De Vido, D.; Mian, C.; Piccolo, M.; Busnardo, B., Medullary thyroid carcinoma: clinical features and long-term follow-up of seventy-eight patients treated between 1969 and 1986. *Thyroid* **1998,** 8, (6), 517-23.

6. Kebebew, E.; Ituarte, P. H.; Siperstein, A. E.; Duh, Q.; Clark, O. H., Medullary thyroid cancer. *Curr Treat Options Oncol* **2000,** 1, (4), 359-67.

7. Modigliani, E.; Cohen, R.; Campos, J. M.; Conte-Devolx, B.; Maes, B.; Boneu, A.; Schlumberger, M.; Bigorgne, J. C.; Dumontier, P.; Leclerc, L.; Corcuff, B.; Guilhem, I., Prognostic factors for survival and for biochemical cure in medullary thyroid carcinoma: results in 899 patients. The GETC Study Group. Groupe d'etude des tumeurs a calcitonine. *Clin Endocrinol (Oxf)* **1998,** 48, (3), 265-73.

8. Da Silva, A. M.; Maciel, R. M.; Da Silva, M. R.; Toledo, S. R.; De Carvalho, M. B.; Cerutti, J. M., A novel germ-line point mutation in RET exon 8 (Gly(533)Cys) in a large kindred with familial medullary thyroid carcinoma. *J Clin Endocrinol Metab* **2003,** 88, (11), 5438-43.

9. Donis-Keller, H.; Dou, S.; Chi, D.; Carlson, K. M.; Toshima, K.; Lairmore, T. C.; Howe, J. R.; Moley, J. F.; Goodfellow, P.; Wells, S. A., Jr., Mutations in the RET proto-oncogene are associated with MEN 2A and FMTC. *Hum Mol Genet* **1993,** 2, (7), 851-6.

10. Dvorakova, S.; Vaclavikova, E.; Duskova, J.; Vlcek, P.; Ryska, A.; Bendlova, B., Exon 5 of the RET proto-oncogene: a newly detected risk exon for familial medullary thyroid carcinoma, a novel germ-line mutation Gly321Arg. *J Endocrinol Invest* **2005,** 28, (10), 905-9.

11. Eng, C.; Clayton, D.; Schuffenecker, I.; Lenoir, G.; Cote, G.; Gagel, R. F.; van Amstel, H. K.; Lips, C. J.; Nishisho, I.; Takai, S. I.; Marsh, D. J.; Robinson, B. G.; Frank-Raue, K.; Raue, F.; Xue, F.; Noll, W. W.; Romei, C.; Pacini, F.; Fink, M.; Niederle, B.; Zedenius, J.; Nordenskjold, M.; Komminoth, P.; Hendy, G. N.; Mulligan, L. M.; et al., The relationship between specific RET proto-oncogene mutations and disease phenotype in multiple endocrine neoplasia type 2. International RET mutation consortium analysis. *JAMA* **1996,** 276, (19), 1575-9.

12. Mulligan, L. M.; Eng, C.; Healey, C. S.; Clayton, D.; Kwok, J. B.; Gardner, E.; Ponder, M. A.; Frilling, A.; Jackson, C. E.; Lehnert, H.; et al., Specific mutations of the RET proto-oncogene are related to disease phenotype in MEN 2A and FMTC. *Nat Genet* **1994,** 6, (1), 70-4.

13. Mulligan, L. M.; Kwok, J. B.; Healey, C. S.; Elsdon, M. J.; Eng, C.; Gardner, E.; Love, D. R.; Mole, S. E.; Moore, J. K.; Papi, L.; et al., Germ-line mutations of the RET proto-oncogene in multiple endocrine neoplasia type 2A. *Nature* **1993,** 363, (6428), 458-60.

14. Eng, C.; Mulligan, L. M.; Healey, C. S.; Houghton, C.; Frilling, A.; Raue, F.; Thomas, G. A.; Ponder, B. A., Heterogeneous mutation of the RET proto-oncogene in subpopulations of medullary thyroid carcinoma. *Cancer Res* **1996,** 56, (9), 2167-70.

15. Marsh, D. J.; Learoyd, D. L.; Andrew, S. D.; Krishnan, L.; Pojer, R.; Richardson, A. L.; Delbridge, L.; Eng, C.; Robinson, B. G., Somatic mutations in the RET proto-oncogene in sporadic medullary thyroid carcinoma. *Clin Endocrinol (Oxf)* **1996,** 44, (3), 249-57.

16. Romei, C.; Elisei, R.; Pinchera, A.; Ceccherini, I.; Molinaro, E.; Mancusi, F.; Martino, E.; Romeo, G.; Pacini, F., Somatic mutations of the ret protooncogene in sporadic medullary thyroid carcinoma are not restricted to exon 16 and are associated with tumor recurrence. *J Clin Endocrinol Metab* **1996,** 81, (4), 1619-22.

17. Siqueira, D. R.; Romitti, M.; da Rocha, A. P.; Ceolin, L.; Meotti, C.; Estivalet, A.; Punales, M. K.; Maia, A. L., The RET polymorphic allele S836S is associated with early metastatic disease in patients with hereditary or sporadic medullary thyroid carcinoma. *Endocr Relat Cancer* **2010,** 17, (4), 953-63.

18. Takahashi, M.; Ritz, J.; Cooper, G. M., Activation of a novel human transforming gene, ret, by DNA rearrangement. *Cell* **1985,** 42, (2), 581-8.

19. Ishizaka, Y.; Itoh, F.; Tahira, T.; Ikeda, I.; Sugimura, T.; Tucker, J.; Fertitta, A.; Carrano, A. V.; Nagao, M., Human ret proto-oncogene mapped to chromosome 10q11.2. *Oncogene* **1989,** 4, (12), 1519-21.

20. Takahashi, M.; Buma, Y.; Hiai, H., Isolation of ret proto-oncogene cDNA with an amino-terminal signal sequence. *Oncogene* **1989,** 4, (6), 805-6.

21. Hofstra, R. M.; Landsvater, R. M.; Ceccherini, I.; Stulp, R. P.; Stelwagen, T.; Luo, Y.; Pasini, B.; Hoppener, J. W.; van Amstel, H. K.; Romeo, G.; et al., A mutation in the RET proto-oncogene associated with multiple endocrine neoplasia type 2B and sporadic medullary thyroid carcinoma. *Nature* **1994,** 367, (6461), 375-6.

22. Myers, S. M.; Eng, C.; Ponder, B. A.; Mulligan, L. M., Characterization of RET proto-oncogene 3' splicing variants and polyadenylation sites: a novel C-terminus for RET. *Oncogene* **1995,** 11, (10), 2039-45.

23. Takahashi, M.; Asai, N.; Iwashita, T.; Isomura, T.; Miyazaki, K.; Matsuyama, M., Characterization of the ret proto-oncogene products expressed in mouse L cells. *Oncogene* **1993,** 8, (11), 2925-9.

24. Jing, S.; Wen, D.; Yu, Y.; Holst, P. L.; Luo, Y.; Fang, M.; Tamir, R.; Antonio, L.; Hu, Z.; Cupples, R.; Louis, J. C.; Hu, S.; Altrock, B. W.; Fox, G. M., GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR-alpha, a novel receptor for GDNF. *Cell* **1996,** 85, (7), 1113-24.

25. Kjaer, S.; Ibanez, C. F., Identification of a surface for binding to the GDNF-GFR alpha 1 complex in the first cadherin-like domain of RET. *J Biol Chem* **2003,** 278, (48), 47898-904.

26. Yu, T.; Scully, S.; Yu, Y.; Fox, G. M.; Jing, S.; Zhou, R., Expression of GDNF family receptor components during development: implications in the mechanisms of interaction. *J Neurosci* **1998,** 18, (12), 4684-96.

27. Santoro, M.; Carlomagno, F.; Romano, A.; Bottaro, D. P.; Dathan, N. A.; Grieco, M.; Fusco, A.; Vecchio, G.; Matoskova, B.; Kraus, M. H.; et al., Activation of RET as a dominant transforming gene by germline mutations of MEN2A and MEN2B. *Science* **1995,** 267, (5196), 381-3.

28. Borrello, M. G.; Smith, D. P.; Pasini, B.; Bongarzone, I.; Greco, A.; Lorenzo, M. J.; Arighi, E.; Miranda, C.; Eng, C.; Alberti, L.; et al., RET activation by germline MEN2A and MEN2B mutations. *Oncogene* **1995,** 11, (11), 2419-27.

29. Carlson, K. M.; Dou, S.; Chi, D.; Scavarda, N.; Toshima, K.; Jackson, C. E.; Wells, S. A., Jr.; Goodfellow, P. J.; Donis-Keller, H., Single missense mutation in the tyrosine kinase catalytic domain of the RET protooncogene is associated with multiple endocrine neoplasia type 2B. *Proc Natl Acad Sci U S A* **1994,** 91, (4), 1579-83.

30. Mulligan, L. M.; Gardner, E.; Smith, B. A.; Mathew, C. G.; Ponder, B. A., Genetic events in tumour initiation and progression in multiple endocrine neoplasia type 2. *Genes Chromosomes Cancer* **1993,** 6, (3), 166-77.

31. Pelizzo, M. R.; Boschin, I. M.; Bernante, P.; Toniato, A.; Piotto, A.; Pagetta, C.; Nibale, O.; Rampin, L.; Muzzio, P. C.; Rubello, D., Natural history, diagnosis, treatment and outcome of medullary thyroid cancer: 37 years experience on 157 patients. *Eur J Surg Oncol* **2007,** 33, (4), 493-7.

32. Mulligan, L. M.; Marsh, D. J.; Robinson, B. G.; Schuffenecker, I.; Zedenius, J.; Lips, C. J.; Gagel, R. F.; Takai, S. I.; Noll, W. W.; Fink, M.; et al., Genotype-phenotype correlation in multiple endocrine neoplasia type 2: report of the International RET Mutation Consortium. *J Intern Med* **1995,** 238, (4), 343-6.

33. Farndon, J. R.; Leight, G. S.; Dilley, W. G.; Baylin, S. B.; Smallridge, R. C.; Harrison, T. S.; Wells, S. A., Jr., Familial medullary thyroid carcinoma without associated endocrinopathies: a distinct clinical entity. *Br J Surg* **1986,** 73, (4), 278-81.

34. Frank-Raue, K.; Buhr, H.; Dralle, H.; Klar, E.; Senninger, N.; Weber, T.; Rondot, S.; Hoppner, W.; Raue, F., Long-term outcome in 46 gene carriers of hereditary medullary thyroid carcinoma after prophylactic thyroidectomy: impact of individual RET genotype. *Eur J Endocrinol* **2006,** 155, (2), 229-36.

35. Machens, A.; Gimm, O.; Hinze, R.; Hoppner, W.; Boehm, B. O.; Dralle, H., Genotype-phenotype correlations in hereditary medullary thyroid carcinoma: oncological features and biochemical properties. *J Clin Endocrinol Metab* **2001,** 86, (3), 1104-9.

36. Punales, M. K.; Graf, H.; Gross, J. L.; Maia, A. L., RET codon 634 mutations in multiple endocrine neoplasia type 2: variable clinical features and clinical outcome. *J Clin Endocrinol Metab* **2003,** 88, (6), 2644-9.

37. Donis-Keller, H., The RET proto-oncogene and cancer. *J Intern Med* **1995,** 238, (4), 319-25.

38. Frank-Raue, K.; Hoppner, W.; Frilling, A.; Kotzerke, J.; Dralle, H.; Haase, R.; Mann, K.; Seif, F.; Kirchner, R.; Rendl, J.; Deckart, H. F.; Ritter, M. M.; Hampel, R.; Klempa, J.; Scholz, G. H.; Raue, F., Mutations of the ret protooncogene in German multiple endocrine neoplasia families: relation between genotype and phenotype. German Medullary Thyroid Carcinoma Study Group. *J Clin Endocrinol Metab* **1996,** 81, (5), 1780-3.

39. Verga, U.; Fugazzola, L.; Cambiaghi, S.; Pritelli, C.; Alessi, E.; Cortelazzi, D.; Gangi, E.; Beck-Peccoz, P., Frequent association between MEN 2A and cutaneous lichen amyloidosis. *Clin Endocrinol (Oxf)* **2003,** 59, (2), 156-61.

40. Milos, I. N.; Frank-Raue, K.; Wohllk, N.; Maia, A. L.; Pusiol, E.; Patocs, A.; Robledo, M.; Biarnes, J.; Barontini, M.; Links, T. P.; de Groot, J. W.; Dvorakova, S.; Peczkowska, M.; Rybicki, L. A.; Sullivan, M.; Raue, F.; Zosin, I.; Eng, C.; Neumann, H. P., Age-related neoplastic risk profiles and

penetrance estimations in multiple endocrine neoplasia type 2A caused by germ line RET Cys634Trp (TGC>TGG) mutation. *Endocr Relat Cancer* **2008,** 15, (4), 1035-41.

41. Frank-Raue, K.; Rybicki, L. A.; Erlic, Z.; Schweizer, H.; Winter, A.; Milos, I.; Toledo, S. P.; Toledo, R. A.; Tavares, M. R.; Alevizaki, M.; Mian, C.; Siggelkow, H.; Hufner, M.; Wohllk, N.; Opocher, G.; Dvorakova, S.; Bendlova, B.; Czetwertynska, M.; Skasko, E.; Barontini, M.; Sanso, G.; Vorlander, C.; Maia, A. L.; Patocs, A.; Links, T. P.; de Groot, J. W.; Kerstens, M. N.; Valk, G. D.; Miehle, K.; Musholt, T. J.; Biarnes, J.; Damjanovic, S.; Muresan, M.; Wuster, C.; Fassnacht, M.; Peczkowska, M.; Fauth, C.; Golcher, H.; Walter, M. A.; Pichl, J.; Raue, F.; Eng, C.; Neumann, H. P., Risk profiles and penetrance estimations in multiple endocrine neoplasia type 2A caused by germline RET mutations located in exon 10. *Hum Mutat* **2011,** 32, (1), 51-8.

42. Gimm, O.; Marsh, D. J.; Andrew, S. D.; Frilling, A.; Dahia, P. L.; Mulligan, L. M.; Zajac, J. D.; Robinson, B. G.; Eng, C., Germline dinucleotide mutation in codon 883 of the RET proto-oncogene in multiple endocrine neoplasia type 2B without codon 918 mutation. *J Clin Endocrinol Metab* **1997,** 82, (11), 3902-4.

43. Smith, D. P.; Houghton, C.; Ponder, B. A., Germline mutation of RET codon 883 in two cases of de novo MEN 2B. *Oncogene* **1997,** 15, (10), 1213-7.

44. Menko, F. H.; van der Luijt, R. B.; de Valk, I. A.; Toorians, A. W.; Sepers, J. M.; van Diest, P. J.; Lips, C. J., Atypical MEN type 2B associated with two germline RET mutations on the same allele not involving codon 918. *J Clin Endocrinol Metab* **2002,** 87, (1), 393-7.

45. Miyauchi, A.; Futami, H.; Hai, N.; Yokozawa, T.; Kuma, K.; Aoki, N.; Kosugi, S.; Sugano, K.; Yamaguchi, K., Two germline missense mutations at codons 804 and 806 of the RET proto-oncogene in the same allele in a patient with multiple endocrine neoplasia type 2B without codon 918 mutation. *Jpn J Cancer Res* **1999,** 90, (1), 1-5.

46. Zedenius, J.; Larsson, C.; Bergholm, U.; Bovee, J.; Svensson, A.; Hallengren, B.; Grimelius, L.; Backdahl, M.; Weber, G.; Wallin, G., Mutations of codon 918 in the RET proto-oncogene correlate to poor prognosis in sporadic medullary thyroid carcinomas. *J Clin Endocrinol Metab* **1995,** 80, (10), 3088-90.

47. Hofstra, R. M.; Fattoruso, O.; Quadro, L.; Wu, Y.; Libroia, A.; Verga, U.; Colantuoni, V.; Buys, C. H., A novel point mutation in the intracellular domain of the ret protooncogene in a family with medullary thyroid carcinoma. *J Clin Endocrinol Metab* **1997,** 82, (12), 4176-8.

48. Bolino, A.; Schuffenecker, I.; Luo, Y.; Seri, M.; Silengo, M.; Tocco, T.; Chabrier, G.; Houdent, C.; Murat, A.; Schlumberger, M.; et al., RET mutations in exons 13 and 14 of FMTC patients. *Oncogene* **1995,** 10, (12), 2415-9.

49. Dabir, T.; Hunter, S. J.; Russell, C. F.; McCall, D.; Morrison, P. J., The RET mutation E768D confers a late-onset familial medullary thyroid carcinoma -- only phenotype with incomplete penetrance: implications for screening and management of carrier status. *Fam Cancer* **2006,** 5, (2), 201-4.

50. Eng, C.; Smith, D. P.; Mulligan, L. M.; Healey, C. S.; Zvelebil, M. J.; Stonehouse, T. J.; Ponder, M. A.; Jackson, C. E.; Waterfield, M. D.; Ponder, B. A., A novel point mutation in the tyrosine kinase domain of the RET proto-oncogene in sporadic medullary thyroid carcinoma and in a family with FMTC. *Oncogene* **1995,** 10, (3), 509-13.

51. Kloos, R. T.; Eng, C.; Evans, D. B.; Francis, G. L.; Gagel, R. F.; Gharib, H.; Moley, J. F.; Pacini, F.; Ringel, M. D.; Schlumberger, M.; Wells, S. A., Jr., Medullary thyroid cancer: management guidelines of the American Thyroid Association. *Thyroid* **2009,** 19, (6), 565-612.

52. Heshmati, H. M.; Gharib, H.; van Heerden, J. A.; Sizemore, G. W., Advances and controversies in the diagnosis and management of medullary thyroid carcinoma. *Am J Med* **1997,** 103, (1), 60-9.

53. Elisei, R.; Cosci, B.; Romei, C.; Bottici, V.; Renzini, G.; Molinaro, E.; Agate, L.; Vivaldi, A.; Faviana, P.; Basolo, F.; Miccoli, P.; Berti, P.; Pacini, F.; Pinchera, A., Prognostic significance of somatic RET oncogene mutations in sporadic medullary thyroid cancer: a 10-year follow-up study. *J Clin Endocrinol Metab* **2008,** 93, (3), 682-7.

54. Moura, M. M.; Cavaco, B. M.; Pinto, A. E.; Domingues, R.; Santos, J. R.; Cid, M. O.; Bugalho, M. J.; Leite, V., Correlation of RET somatic mutations with clinicopathological features in sporadic medullary thyroid carcinomas. *Br J Cancer* **2009,** 100, (11), 1777-83.

55. Beldjord, C.; Desclaux-Arramond, F.; Raffin-Sanson, M.; Corvol, J. C.; De Keyzer, Y.; Luton, J. P.; Plouin, P. F.; Bertagna, X., The RET protooncogene in sporadic pheochromocytomas: frequent MEN 2-like mutations and new molecular defects. *J Clin Endocrinol Metab* **1995,** 80, (7), 2063-8.

56. van der Harst, E.; de Krijger, R. R.; Bruining, H. A.; Lamberts, S. W.; Bonjer, H. J.; Dinjes, W. N.; Proye, C.; Koper, J. W.; Bosman, F. T.; Roth, J.; Heitz, P. U.; Komminoth, P., Prognostic value of RET proto-oncogene point mutations in malignant and benign, sporadic phaeochromocytomas. *Int J Cancer* **1998,** 79, (5), 537-40.

57. Robledo, M.; Gil, L.; Pollan, M.; Cebrian, A.; Ruiz, S.; Azanedo, M.; Benitez, J.; Menarguez, J.; Rojas, J. M., Polymorphisms G691S/S904S of RET as genetic modifiers of MEN 2A. *Cancer Res* **2003,** 63, (8), 1814-7.

58. Wiench, M.; Wygoda, Z.; Gubala, E.; Wloch, J.; Lisowska, K.; Krassowski, J.; Scieglinska, D.; Fiszer-Kierzkowska, A.; Lange, D.; Kula, D.; Zeman, M.; Roskosz, J.; Kukulska, A.; Krawczyk, Z.; Jarzab, B., Estimation of risk of inherited medullary thyroid carcinoma in apparent sporadic patients. *J Clin Oncol* **2001,** 19, (5), 1374-80.

59. Eng, C.; Mulligan, L. M.; Smith, D. P.; Healey, C. S.; Frilling, A.; Raue, F.; Neumann, H. P.; Pfragner, R.; Behmel, A.; Lorenzo, M. J.; et al., Mutation of the RET protooncogene in sporadic medullary thyroid carcinoma. *Genes Chromosomes Cancer* **1995,** 12, (3), 209-12.

60. Gimm, O.; Neuberg, D. S.; Marsh, D. J.; Dahia, P. L.; Hoang-Vu, C.; Raue, F.; Hinze, R.; Dralle, H.; Eng, C., Over-representation of a germline RET sequence variant in patients with sporadic medullary thyroid carcinoma and somatic RET codon 918 mutation. *Oncogene* **1999,** 18, (6), 1369-73.

61. Elisei, R.; Cosci, B.; Romei, C.; Bottici, V.; Sculli, M.; Lari, R.; Barale, R.; Pacini, F.; Pinchera, A., RET exon 11 (G691S) polymorphism is significantly more frequent in sporadic medullary thyroid carcinoma than in the general population. *J Clin Endocrinol Metab* **2004,** 89, (7), 3579-84.

62. Rocha, A. P.; Magalhães, P. R.; Maia, A. L.; Maciel, L. Z., Polimorfismos Genéticos: Implicações na Patogênese do Carcinoma Medular de Tireóide. *Arq Bras Endocrinol Metab* **2007,** 5, (51), 723-730.

63. Cebrian, A.; Lesueur, F.; Martin, S.; Leyland, J.; Ahmed, S.; Luccarini, C.; Smith, P. L.; Luben, R.; Whittaker, J.; Pharoah, P. D.; Dunning, A. M.; Ponder, B. A., Polymorphisms in the initiators of RET (rearranged during transfection) signaling pathway and susceptibility to sporadic medullary thyroid carcinoma. *J Clin Endocrinol Metab* **2005,** 90, (11), 6268-74.

64. Fugazzola, L.; Muzza, M.; Mian, C.; Cordella, D.; Barollo, S.; Alberti, L.; Cirello, V.; Dazzi, D.; Girelli, M. E.; Opocher, G.; Beck-Peccoz, P.; Persani, L., RET genotypes in sporadic medullary thyroid cancer: studies in a large Italian series. *Clin Endocrinol (Oxf)* **2008,** 69, (3), 418-25.

65. Lesueur, F.; Cebrian, A.; Robledo, M.; Niccoli-Sire, P.; Svensson, K. A.; Pinson, S.; Leyland, J.; Whittaker, J.; Pharoah, P. D.; Ponder, B. A., Polymorphisms in RET and its coreceptors and ligands as genetic modifiers of multiple endocrine neoplasia type 2A. *Cancer Res* **2006,** 66, (2), 1177-80.

66. Baumgartner-Parzer, S. M.; Lang, R.; Wagner, L.; Heinze, G.; Niederle, B.; Kaserer, K.; Waldhausl, W.; Vierhapper, H., Polymorphisms in exon 13 and intron 14 of the RET protooncogene: genetic modifiers of medullary thyroid carcinoma? *J Clin Endocrinol Metab* **2005,** 90, (11), 6232-6.

67. Sharma, B. P.; Saranath, D., RET gene mutations and polymorphisms in medullary thyroid carcinomas in Indian patients. *J Biosci* **2011,** 36, (4), 603-11.

68. Sromek, M.; Czetwertynska, M.; Skasko, E.; Zielinska, J.; Czapczak, D.; Steffen, J., The frequency of selected polymorphic variants of the RET gene in patients with medullary thyroid carcinoma and in the general population of central Poland. *Endocr Pathol* **2010,** 21, (3), 178-85.

69. Wohllk, N.; Soto, E.; Bravo, M.; Becker, P., Polimorfismos G691S, L769L y S836S del proto-oncogen RET no se asocian a mayor riesgo de cáncer medular tiroideo esporádico en pacientes chilenos. *Rev Méd Chile* **2005,** 133, (397-402).

70. Magalhães, P. K.; de Castro, M.; Elias, L. L.; Soares, E. G.; Maciel, L. M., Polymorphisms in the RET proto-oncogene and the phenotypic presentation of familial medullary thyroid carcinoma. *Thyroid* **2004,** 14, (10), 848-52.

71. Berard, I.; Kraimps, J. L.; Savagner, F.; Murat, A.; Renaudin, K.; Nicolli-Sire, P.; Bertrand, G.; Moisan, J. P.; Bezieau, S., Germline-sequence variants S836S and L769L in the RE arranged during Transfection (RET) proto-oncogene are not associated with predisposition to sporadic medullary carcinoma in the French population. *Clin Genet* **2004,** 65, (2), 150-2.

72. Ruiz, A.; Antinolo, G.; Fernandez, R. M.; Eng, C.; Marcos, I.; Borrego, S., Germline sequence variant S836S in the RET proto-oncogene is associated with low level predisposition to sporadic medullary thyroid carcinoma in the Spanish population. *Clin Endocrinol (Oxf)* **2001,** 55, (3), 399-402.

73. Tamanaha, R.; Camacho, C. P.; Pereira, A. C.; da Silva, A. M.; Maciel, R. M.; Cerutti, J. M., Evaluation of RET polymorphisms in a six-generation family with G533C RET mutation: specific RET variants may modulate age at onset and clinical presentation. *Clin Endocrinol (Oxf)* **2009,** 71, (1), 56-64.

74. Griseri, P.; Sancandi, M.; Patrone, G.; Bocciardi, R.; Hofstra, R.; Ravazzolo, R.; Devoto, M.; Romeo, G.; Ceccherini, I., A single-nucleotide polymorphic variant of the RET proto-oncogene is underrepresented in sporadic Hirschsprung disease. *Eur J Hum Genet* **2000,** 8, (9), 721-4.

75. Borrego, S.; Wright, F. A.; Fernandez, R. M.; Williams, N.; Lopez-Alonso, M.; Davuluri, R.; Antinolo, G.; Eng, C., A founding locus within the RET proto-oncogene may account for a large proportion of apparently sporadic Hirschsprung disease and a subset of cases of sporadic medullary thyroid carcinoma. *Am J Hum Genet* **2003,** 72, (1), 88-100.

76. Lu, H.; Huan, C., Transcription factor NFAT, its role in cancer development, and as a potential target for chemoprevention. *Curr Cancer Drug Targets* **2007,** 7, (4), 343-53.

Parte II

The *RET* polymorphic allele S836S is associated with early metastatic disease in patients with hereditary or sporadic medullary thyroid carcinoma

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THE *RET* POLYMORPHIC ALLELE S836S IS ASSOCIATED WITH EARLY METASTATIC DISEASE IN PATIENTS WITH HEREDITARY OR SPORADIC MEDULLARY THYROID CARCINOMA

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Running title: *RET* polymorphisms and MEN 2A

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Abstract

The possible role of *RET* variants in modifying the natural course of medullary thyroid carcinoma (MTC) is still a matter of debate. Here, we investigate whether the *RET* variants L769L, S836S, and G691S/S904S influence disease presentation in hereditary or sporadic MTC patients. One hundred and two patients with hereditary MTC and 81 patients with sporadic MTC attending our Institution were evaluated. The frequencies of *RET* polymorphisms in hereditary MTC were as follows: L769L, 17.3%, S836S, 7.95% and S904S/G691S, 18.2%. No associations were observed between these polymorphisms and pheochromocytoma, hyperparathyroidism, lymph-node or distant metastases. However, patients harboring the S836S variant were younger than those without this allele (17±8.2*vs*.28.6±14.4 years, P=0.01), suggesting that these patients had metastases at young age. Accordingly, the cumulative frequency of local and/or distant metastases as estimated by Kaplan-Meier curves showed that lymph-nodes and distant metastases occurred earlier in patients harboring S836S variant (P=0.003 and P=0.026, respectively). The S836S allele frequency was higher in sporadic MTC patients than in controls (10.5vs.3.1%, P=0.01). Individuals harboring S836S variant were younger (38.6±13.3*vs*.48.5±16.7 years, P=0.02) and showed a higher percentage of lymph node and distant metastasis (P=0.02 and P=0.04, respectively). Kaplan-Meier estimates of lymph-node and distant metastasis yielded distinct curves for patients with or without the S836S allele (P=0.002 and P=0.001, respectively). Additional analyses using a COX regression model showed that the S836S variant was independently associated with metastatic disease [HR 2.82 (95 %CI 1.51-5.26), P=0.001]. In conclusion, the *RET* S836S variant is associated with early onset and increased risk for metastatic disease in patients with hereditary or sporadic MTC.

Introduction

Medullary thyroid carcinoma (MTC), a malignant neoplasia of the parafollicular C cells of the thyroid, may occur sporadically or as part of the inherited cancer syndrome of multiple endocrine neoplasia type 2 (MEN 2) (Kouvaraki, et al. 2005). The MEN 2 syndrome includes three clinically distinct forms: MEN 2A, MEN 2B, and familial medullary thyroid carcinoma (FMTC). In patients with FMTC, only the thyroid is affected. Patients with MEN 2A develop MTC, pheochromocytoma (PHEO) and/or primary hyperparathyroidism (HPT). MEN 2B patients have MTC, PHEO, ganglioneuromas of the digestive tract, mucosal neuromas, and / or skeletal abnormalities.

The *RET* protooncogene is the susceptibility gene for hereditary MTC and recent studies showed a time-dependent MTC progression, strengthening the importance of DNA-based *RET* genotype analysis for identification of asymptomatic gene carriers at risk of developing MTC (Machens, et al. 2003; Punales, et al. 2008). Gain-of-function germline mutations in MEN 2A and FMTC syndromes have been described in *RET* exons 5, 8, 10, 11, 13, 14, and 15 (Kouvaraki et al. 2005). However, the majority of MEN 2A families have mutations of one of the five conserved cysteine residues in exon 10 (codons 609, 611, 618, and 620) or exon 11 (codon 634) in the extracellular domain of RET (Eng, et al. 1996; Ponder 1999). The presence of any mutation at codon 634 has been associated with the presence of PHEO and HPT. Conversely, mutations at codons 768 and 804 are thus far associated with FMTC. The reasons for the genotype-phenotype correlations have not been completely clarify yet. Although the different levels of RET activation induced by the different mutations could partially explain the phenomena, the observed clinical variability and aggressiveness in members of the same family suggest a role for genetic modifiers in the clinical course of MTC (Ponder 1999; Machens, et al. 2001; Robledo, et al. 2003).

The possible role of neutral *RET* sequence variants in modifying the MEN 2 clinical course or MEN 2-related tumors is still a matter of debate. Some studies have shown that *RET* single nucleotide polymorphisms (SNPs) could interfere in the disease presentation of hereditary MTC syndromes (Magalhaes, et al. 2004; Rocha, et al. 2007). Robledo et al. demonstrated that two of these *RET* variants (G691S and S904S) may modify the age at onset of MTC tumor in family members (Robledo et al. 2003), although these findings could not be replicated in a large European population sample (Lesueur, et al. 2006). It was also suggested that the L769L polymorphism might contribute to the earlier onset of MTC in a patient with a V804M mutation (Magalhaes et al. 2004). Recently, an association was described between two *RET* variants, IVS1-126G>T and

[IVS8+82A>G; 85_86insC], with the clinical course of hereditary MTC in a six-generation family with a G533C *RET* mutation (Tamanaha, et al. 2009). Nevertheless, other studies failed to demonstrate any effect of *RET* polymorphisms on the natural course of hereditary MTC (Fernandez, et al. 2006a; Lesueur et al. 2006).

The purpose of this study was to investigate whether the *RET* neutral variants G691S, L769L, S836S, or S904S influence the clinical presentation and disease outcome in a large cohort of individuals with MEN 2A. These polymorphisms were selected based on their previous association with clinical course of hereditary or sporadic MTC (Robledo et al. 2003; Wiench, et al. 2004; Baumgartner-Parzer, et al. 2005; Lesueur et al. 2006). We observed that the S836S polymorphism was associated with younger age at diagnosis and early metastatic disease in hereditary disease. Therefore, we have decided to evaluate whether these findings would be evident in sporadic MTC.

Material and Methods

Patients

Patients with a diagnosis of hereditary MTC attending the Endocrine Division at Hospital de Clínicas de Porto Alegre were invited to participate in the study. Since 1997, our division has been a reference center for molecular testing of *RET* germ line mutations in Brazil, and therefore patients referred to us by other Brazilian centers for molecular investigation were also invited to participate. All patients and/or their legal guardians provided written consent in accordance with the institutional Ethics Committee.

The data collected for each individual included the clinical characteristics of family members (association of other endocrine neoplasias), the type of *RET* mutations, and information on atypical features noted, such as Hirschsprung's disease or cutaneous lichen amyloidosis (CLA). Patients underwent a complete clinical examination, laboratory tests [levels of basal calcitonin (Until December 2003, Calcitonin IRMA-DSL7700, Diagnostic Systems Laboratories, Inc., Webster, TX, reference range less than 10 pg/ml and, after January 2004, Immulite 2000, Diagnostic Products Corporation, Los Angeles, CA, USA; VR. Male < 12.0pg/ml and female < 6.0 pg/m)], plasma PTH (Immulite 2000 Intact PTH, Diagnostic Products, Los Angeles, CA, USA), urinary fractionated metanephrines (HPLC), and diagnostic imaging investigation that included cervical ultrasonography, thorax and abdominal computed tomography (CT). Selected patients were

submitted to whole-body metaiodobenzylguanidine scintigraphy to rule out PHEO and/or local and distant metastasis.

Our initial sample comprised 102 patients, with germ line mutations of the *RET* protooncogene and/or immunohistochemistry diagnosis of MTC, who were identified by genetic screening at our institution, belonging to 17 unrelated families with MEN 2A and its variants or FMTC. Of them, 68 patients were diagnosed based on clinical evidence of disease, and 34 gene carriers identified through genetic screening. Subjects who presented physical signs compatible with MTC (palpable thyroid nodule and/or lymph node enlargement) at diagnosis were considered as presenting clinical disease and individuals without physical disease were considered as asymptomatic gene carriers. Fourteen of these patients were excluded, either because they were awaiting surgery (2 patients) or not enough material was available for polymorphism analysis (11 patients). We also excluded one patient with a mutation at RET codon 768, exon 13, Glu→Asp (E768D) due to the characteristic low-risk disease phenotype.

We also evaluated 81 patients with sporadic MTC. The diagnosis of sporadic MTC was based on the histopathological / immunohistochemistry findings and absence of known germ line RET point mutations in exons 8, 10, 11 or 13–16. The clinical and laboratorial data were collected for each individual in the sporadic group. A group of 80 health volunteers attending the blood-donation facility of Hospital de Clínicas de Porto Alegre (Porto Alegre, Brazil) constituted our control group. A standard questionnaire was used to collect information about age, sex, skin color, and history of neoplasias.

Total thyroidectomy was performed in all patients with varying cervical neck dissection procedures. The diagnosis of lymph node metastasis was based on histological examination. Patients with suspicious distant metastasis (i.e. the presence of local metastasis and/or serum calcitonin > 150 pg/ml) underwent imaging exams (cervical, thoracic and abdomen CT – or liver magnetic resonance imaging- and bone scintigraphy). Patients with undetectable calcitonin levels were considered free of disease.

Patients with PHEO or HPT underwent specific surgery. Tumor staging was performed according to the current International Union against Cancer (UICC) TNM classification (O'Sullivan and Shah 2003).

Single nucleotide polymorphism analysis

The following *RET* SNPs were selected based on their previous association with the clinical course of sporadic or hereditary MTC: G691S (codon 691 of exon 11, GlyGGT→SerAGT), L769L

(codon 769 of exon 13, LeuCTT→LeuCTG), S836S (codon 836 of exon 14, SerAGC→SerAGT), and S904S (codon 904 of exon 15, SerTCC→SerTCG). For genotyping, genomic DNA was prepared from peripheral blood leukocytes by standard procedures, and the fragments covering the *RET* variants were amplified using PCR primers and conditions previously described (Punales, et al. 2003). Genotyping was performed using either restriction fragment length polymorphism (RFLP) or direct sequencing. For RFLP analysis, an aliquot of PCR product was digested with the appropriate restriction enzyme and analyzed as previously described (Punales et al. 2003). For sequencing, PCR products were purified using the GFX PCR DNA purification kit (GE Healthcare, Buckinghamhire, UK) and submitted to direct sequencing using the Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA).

Somatic M918T mutation analysis

For sporadic patients, we analyzed the frequency of somatic M918T mutation. The MTC samples were material paraffin-embedded formalin-fixed tissue blocks. DNA was extracted using the Magnesil Genomic Fixed Tissue System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Exon 16 was amplified by PCR using 100 to 300ng/µl of DNA in a reaction Mix (25µl) contained 20 mM Tris-HCl pH 8.0, 50 mM KCl, 2 mM MgCl2, 0,2 mM dNTPs, 0.2 mM of each primer, and 1.25 U Platinum Taq DNA Polymerase (Invitrogen Life Technology, Carlsbad, CA, USA). The running profile of the amplification and RFLP analysis were similar to those described for genomic DNA (Punales et al. 2003).

Statistical analysis

Results are expressed as mean ± SD or median and interquartile intervals unless otherwise specified. Baseline characteristics were compared using the Chi-Square test or Fisher's exact test for qualitative variables, or the Student's t test or Mann-Whitney's *U* test for quantitative variables. Hardy-Weinberg equilibrium for each SNP was assessed by the Fisher's exact test. The differences in cumulative lymph node and/or distant metastasis between groups were tested by Kaplan-Meier curves; comparisons between curves were performed using the log rank test. We performed a Cox regression to investigate the effect of several variables upon the time of a specified event: the presence of metastasis. The Statistical Package for the Social Sciences 15.0 (SPSS Inc., Chicago, IL) was used and $P<0.05$ was considered as statistically significant.

Results

Frequency of RET polymorphisms in MEN 2A patients

Table 1 shows the clinical and molecular data of the families with MEN 2A. Of the 17 independent families with hereditary MTC analyzed, 13 were classified as MEN 2A, 3 classified as MEN 2A associated with CLA and 1 associated with HIRS and 1 classified as FMTC. All but four MEN 2A/FMTC kindred had a mutation at *RET* codon 634 in exon 11, the most prevalent mutation accounting for 89 % of cases. The identified mutations were as follows: Cys→Tyr (C634Y, 65.9%), Cys→Arg (C634R, 15.4%), and Cys→Trp (C634W 7.7%).

Table 1. Clinical presentation and *RET* germline mutations in multiple endocrine neoplasia 2A patients

Phenotype	**N families**	***RET* mutation**	**Affected Individuals**	**CCH**	**MTC**	**PHEO**	**HPT**
MEN 2A	6	C634Y	33	2	31	11	5
	4	C634R	8		8	5	3
	1	C634W	7		7	3	3
	2	C618R	8		8	2	0
MEN 2A+	2	C634R	6		6	2	0
CLA	1	C634Y	25		25	3	5
MEN 2A + HIRS	1	C618R	1		1	1	0
TOTAL	17		88	2	86	27	16

MTC, medullary thyroid carcinoma; PHEO, pheochromocytoma; HPT, hyperparathyroidism; CCH, C-Cell hyperplasia; CLA, cutaneous lichen amyloidosis; HIRS, Hirschsprung's disease

The allele frequencies of the *RET* polymorphism are shown in Table 2. The observed SNP frequencies were similar to those reported in the literature (Elisei, et al. 2004; Wiench et al. 2004; Baumgartner-Parzer et al. 2005; Wohllk N 2005; Severskaia, et al. 2006). Confirming previous

studies, the two variants G691S and S904S were in linkage disequilibrium and, therefore, to avoid redundant information, the results were grouped together and referred as G691S-S904S (Robledo et al. 2003; Elisei et al. 2004; Tamanaha et al. 2009). All genotypes analyzed were in Hardy-Weinberg equilibrium (P>0.20).

Table 2. Frequency of RET polymorphisms in Multiple Endocrine Neoplasia 2A patients (n=88)

Sequence Variant		Genotype distribution			Allele frequency (%)	Prevalence in literature * (%)
Exon		Wild type	Heterozygous	Homozygous		
13	L769L	58	26	4	17.3	21.6-31
14	S836S	74	14	0	7.95	1-16
15	G691S/S904S	59	26	3	18.2	4.5-27

Hardy-Weinberg equilibrium Chi-square P> 0.20

*ref. Baumgartner-Parzer, Lang et al. 2005; Wiench, Wloch et al. 2004; Elisei, Cosci et al. 2004 Wohllk, Soto 2005; Severskaia, Saenko et al. 2006.

Clinical and oncological features of hereditary MTC patients

The clinical and oncological features of the subjects are listed in Table 3. The median basal serum calcitonin level at diagnosis was 140 (30-988.6) pg/ml. At first, we assessed whether the polymorphisms could have an effect on the age at onset of disease. Analysis of *RET* variants failed to demonstrated differences in the age of diagnosis related to the presence or the absence of the L769L or G691S/ S904S polymorphic allele. However, patients harboring the S836S variant were significantly younger than those without this allele (17.0±8.2 *vs*. 28.6±14.4 years, P=0.01). There were no differences in basal serum calcitonin at diagnosis between individuals with or without S836S polymorphic allele [15 (8-34) *vs* 152 (36.9-1025] pg/ml, P=0.25).

It is reasonable to speculate that in those patients identified through molecular diagnosis, the natural course of the disease was interrupted by intervention, and the age of diagnosis would be lower than that observed in those individuals in whom the disease evolved naturally. This could be a confusing factor, particularly when analyzing age at onset of the disease. Therefore, we also analyzed both groups separately to avoid selection bias. Table 4 shows the effect of *RET* polymorphisms on MTC presentation in individuals diagnosed by genetic screening or clinical

evidence of disease. There were no differences in age or serum calcitonin levels [37.5 (9.9-73) *vs* 9.5 (7.1-24.1) pg/ml, P=0.11] at diagnosis between individuals with or without polymorphic alleles in the group diagnosed by genetic screening. However, the group of patients with clinical evidence of disease at diagnosis with the polymorphism S836S were younger than those without this allele (20.7±8.1 *vs*. 33.3±13.1 years, P=0.03). No significant differences were observed in serum calcitonin levels [540 (103.5-1800) *vs* 377.4 (344-410.9) pg/ml, P=0.73].

Table 3. Clinical and oncological features of multiple endocrine neoplasia 2A patients according to the presence of the polymorphic allele.

Patients (n)	**Total** (88)	**WT** (58)	**L769L** (30)	**P**	**WT** (74)	**S836S** (14)	**P**	**WT** (59)	**G691S/S904S** (29)	**P**
Sex female (%)	56	60.3	56.7	0.91*	62.2	42.9	0.29*	55.9	65.5	0.53*
Age[1]	27.6±15.8	28.3±14.9	25.1±13.1	0.34	28.6±14.4	17.0± 8.2	0.01	28.3±15.4	25.0±12.1	0.34
Pheo (%)	27	20.7	36.7	0.22*	27.0	21.4	0.81**	25.4	27.6	0.34*
HPT (%)	17	15.5	26.7	0.36*	18.9	21.4	0.89**	22.0	13.8	0.25*
PN1[2](%)	33	31.3	41.4	0.51*	34.8	36.4	1.0**	40.8	25	0.25*
PM1[2] (%)	13	10.4	13.8	0.72**	12.1	9.1	1.0**	10.2	14.3	0.71**

Pheo, pheochromocytoma; HPT, hyperparathyroidism; PN1, lymph node metastasis; PM1, distant metastasis; WT, Wild-type;
[1]Age, age at diagnosis, expressed as mean ± SD. Variables were compared using Student's t test.
[2]Data available for only 79 patients
Qualitative variables were compared using the Yates' Chi-Square test* or Fisher's exact test

Table 4. Clinical and oncological features of multiple endocrine neoplasia 2A patients diagnosed based on genetic screening or clinical grounds

Patients (n)	**Total** (33)	**WT** (23)	**L769L** (10)	**P**	**WT** (25)	**S836S** (8)	**P**	**WT** (25)	**G691S/S904S** (8)	**P**
Genetic screening										
Sex female (%)	52.9	52.2	60	0.72**	60	37.5	0.41**	52	62.5	0.70**
Age[1]	14.0±7.1	14.4±3.9	14.4±10.4	0.98	14.7±7.3	13.5±6.5	0.71	14.4±7.3	14.3±6.7	0.98
(n)	(55)	(35)	(20)	**P**	(49)	(6)	**P**	(34)	(21)	**P**
Clinical disease										
Sex female (%)	58.2	65.7	55	0.62*	63.3	50	0.66**	58.8	66.7	0.77*
Age[1]	32.1±13.1	33.6±14.0	29.8±11.4	0.31	33.3±13.1	20.7±8.1	0.03	34.5±13.8	28.6±11.3	0.15
Pheo (%)	40.3	34.3	55	0.23*	40.8	50	0.87**	44.1	38.1	0.36*
HPT (%)	23.9	22.9	40	0.29*	26.5	50	0.47**	35.3	19	0.22*
PN1[2] (%)	53	42.9	60	0.34*	46.9	66.7	0.42**	58.8	33.3	0.11*
PM1[2] (%)	19.4	14.3	20	0.71**	16.3	16.7	1.00**	14.7	19	0.72**

Pheo, pheochromocytoma; HPT, hyperparathyroidism; PN1, lymph node metastasis; PM1, distant metastasis; WT, Wild-type.
Age[1], age at diagnosis, expressed as mean ± SD. Variables were compared using Student's t test.
[2]Data available for only 46 patients.
Qualitative variables were compared using the Yates' Chi-Square test* or Fisher's exact test**

None of the *RET* polymorphisms were associated with the presence of PHEO, HPT, lymph node or distant metastasis at diagnosis (Table 3). Thus, it was somewhat puzzling that patients heterozygous for the S836S allele, on average 11 years younger than wild-type subjects, presented a virtually identical percentage of lymph node, and distant metastasis. These results suggested to us that these events occurred earlier in individuals harboring the S836S genotype. To test this hypothesis, we have used the Kaplan-Meier model. As gene dysfunction is present since birth, we assumed that the individual age at surgery would indicate the period of exposure. Kaplan-Meier estimates of cumulative lymph node and distant metastasis yielded distinct curves for patients harboring the S836S allele (P=0.003 and P=0.026, respectively, Figure 1). Kaplan-Meier analysis of cumulative metastasis for L769L and G691S/ S904S genotypes yielded similar curves (data not shown).

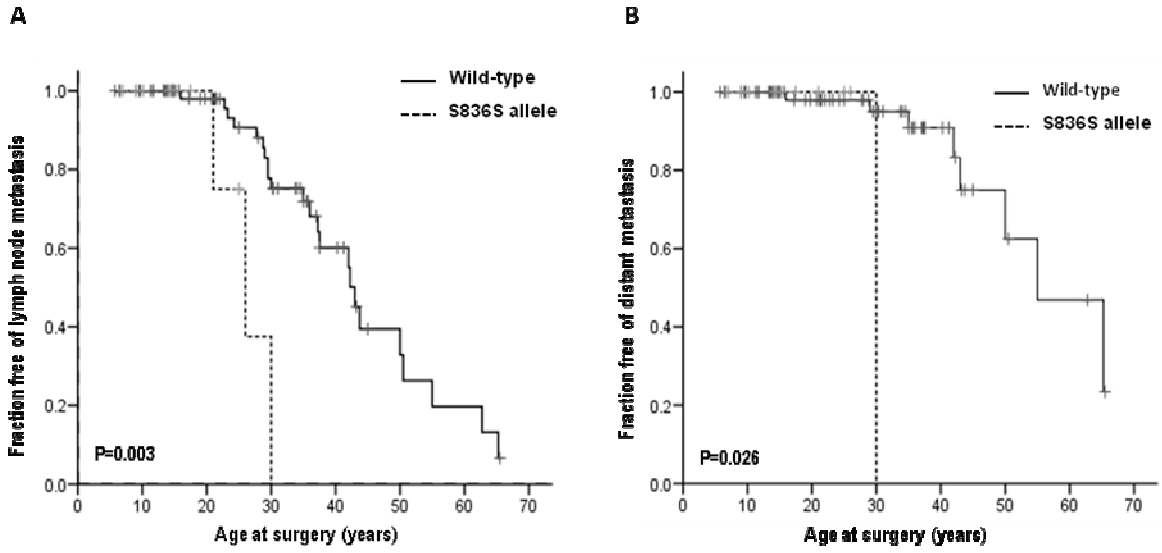

Figure 1. Kaplan-Meier estimates of the proportion of hereditary MTC patients (n=79) with lymph node (A) or distant metastases (B) at diagnosis. *The log rank test was used to compare curves.

RET polymorphisms in sporadic MTC patients

Next, we evaluated the allele frequency of the S836S polymorphism in 81 patients with sporadic MTC followed at our Institution. Table 5 shows the clinical characteristics of the studied patients. The median basal serum calcitonin level at diagnosis was 682 (67.7-2650) pg/ml. The control group consisted of 80 blood donor volunteers. The median age was 48.2 ± 10.1 years (P=0.41), and the percentage of women was 59.3% (P=0.20). None had a recorded history of

malignancy or endocrine disease. The ethnic background of both cases and controls was similar, with more than 95% of Caucasians. The frequency of the S836S allele was higher in sporadic MTC patients when we compared with controls (10.5% *vs.* 3.2%, P= 0.01) but similar to that observed in the hereditary group (7.95%, P=0.43). Genotypes were in Hardy-Weinberg equilibrium (P>0.20).

Table 5. Clinical and oncological features of sporadic MTC patients according to the presence of the S836S polymorphic allele

Patients (n)	**Total** (81)	**WT** (64)	**S836S** (17)	P
Sex female (%)	59.3	59.4	58.8	1.0*
Age[1]	27.6±15.8	48.5±16.7	38.6±13.3	0.02
PN1[2] (%)	50.6	45.9	81.3	0.02*
PM1[2] (%)	22.2	17.7	43.8	0.04**

PN1, lymph node metastasis; PM1, distant metastasis; WT, wild-type;
[1]Age, age at diagnosis, expressed as mean±SD. Variables were compared using Student's t test.
[2] Data available for 74 patients.
Qualitative variables were compared using the Yates' Chi-Square test* or Fisher's exact test**

Of the 81 MTC patients, 64 (79%) were homozygous for the wild-type allele (CC) and 17 patients (21%) were heterozygous for S836S polymorphic allele. There was no homozygosis for the S836S polymorphic allele. Individuals harboring the S836S variant were significantly younger at diagnosis than those without this allele (38.6±13.3 *vs.* 48.5±16.7 years, P=0.02), has higher serum calcitonin level [4193 (1600-13737) *vs* 539 (28.2-1440) pg/ml, P=0.005] and presented a higher percentage of lymph-node and distant metastasis at diagnosis (81.3% *vs.* 45.9% and 43.8% *vs.* 17.7%, P=0.02 and P= 0.04, respectively). Accordingly, Kaplan-Meier estimates of cumulative lymph node and distant metastasis yielded distinct curves for patients with or without the S836S allele (P=0.002 and P=0.001, respectively, Figure 2), further demonstrating that metastatic disease occurred earlier in those individuals harboring the S836S variant.

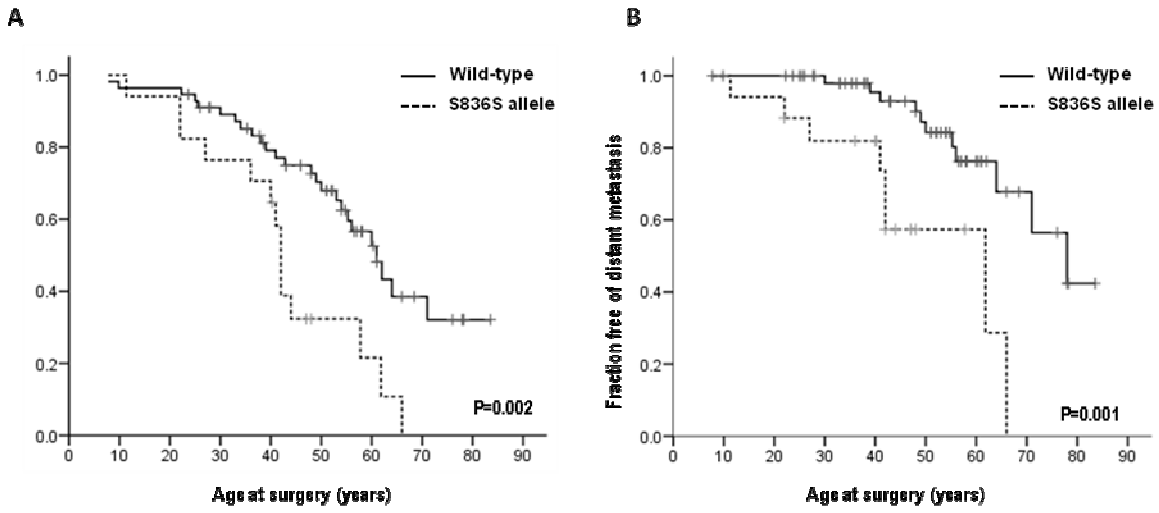

Figure 2. Kaplan-Meier estimates of the proportion of sporadic MTC patients (n=74) with lymph node (A) or distant metastases (B) at diagnosis. * The log rank test was used to compare curves.

Somatic M918T mutation analysis

The following step was to look for somatic *RET* M918T mutation in sporadic MTC group, since it has been shown that the presence of this missense somatic *RET* mutation correlates with the presence of lymph node metastases at diagnosis (Elisei, et al. 2008) and could be a confounding factor in our analysis.

Forty paraffin-embedded MTC samples were available. Of them, we were unable to extract DNA from nine samples even after several repeated attempts. Of the 31 DNA samples available for analysis, 25 (80.6%) were found to have somatic M918T. Although we observed a higher frequency of the S836S allele in this subgroup of patients (11/31, 35.5%), there was no significant association between somatic M918T mutation and S836S polymorphic allele (10/25 (40%) vs. 1/6 (16.7%), respectively, P=0.38).

S836S polymorphism is an independent risk factor for metastatic disease in MTC patients

Because previous studies demonstrated that specific nucleotide and amino acid exchanges at codon 634 might have a direct impact on tumor aggressiveness in MEN 2A (Punales et al. 2003; Milos, et al. 2008), we speculated whether co-segregation with a specific *RET* mutation could interfere our results.

According with the germline mutation, the 14 patients harboring the S836S polymorphic allele were distributed as following: 10 out of 62 individuals with C634Y mutation (3 kindred) and 4 out of 7 individuals with C634W (1 kindred). We found that in all cases the S836S variant did not co-segregate with the germline mutation and was inherited from the unaffected parent.

To further evaluate whether the effect of S836S variant allele was associated with a specific germline mutation, we used the Cox proportional hazard survival analysis with the presence of metastasis at diagnosis as the outcome and the survival time as the age at diagnosis of MTC. To increase the statistical power of the analysis, all patients with MTC diagnosis (hereditary and sporadic groups) were included (n=153). The results are shown in Table 6. The presence of the neutral *RET* S836S variant was an independent risk factor for early local or distant metastatic disease MTC. As expected, all germline mutations except C618R were significantly associated with increased risk for early metastatic disease (Table 6). The lack of significance in the C618R association was probably due to the small number of subjects harboring this mutation (nine individuals).

Table 6. Analysis of survival Cox regression (n=153)

Variables	*b*	P	HR	CI
S836S	1.04	0.001	2.82	(1.51-5.26)
C634W	1.79	0.007	6.03	(1.63-22.4)
C634Y	0.76	0.014	2.14	(1.17-3.93)
C634R	1.90	< 0.005	6.69	(2.45-18.27)
C618R	0.67	0.27	1.96	(0.59-6.51)

b: regression coefficient; HR: hazard ratio; CI: confidence interval.

Discussion

In the present study, we have demonstrated that the neutral *RET* polymorphism S836S is associated with early onset and increased risk for metastatic disease at a younger age in individuals with hereditary or sporadic MTC. Patients harboring the variant allele were younger at diagnosis and presented with early local and distant metastasis, as assessed by the Kaplan Meyer model. Moreover, additional analysis using multivariate Cox regression identified this polymorphism as an independent risk factor for lymph node or distant metastasis.

Several SNPs of the *RET* protooncogene have been described in the general population as well as in patients with familial and sporadic MTC (Gimm, et al. 1999; Ruiz, et al. 2001; Elisei et al. 2004; Severskaia et al. 2006; Tamanaha et al. 2009). Here, we have studied the frequency of the exonic *RET* polymorphisms L769L**,** S836S and G691S/ S904S in 17 MEN 2A families. We did not

detect significant differences related to disease phenotype or tumor stage. However, we observed that patients harboring the S836S variant allele were about 11 years younger than those with the wild type genotype (Table 3). To rule out selection bias on the age of diagnosis due to genetic screening, patients were analyzed separately based on the presence or absence of clinical disease at diagnosis. Of particular note, no differences in age at diagnosis in the group were diagnosed by genetic screening but the positive association between the polymorphism S836S and earlier age at diagnosis remained in the group with clinical evidence of disease at diagnosis (Table 4). Surprisingly, despite that difference in age at diagnosis, patients with or without the polymorphic allele displayed a virtually identical percentage of lymph node and distant metastases (Table 3). Thus, we speculated that these individuals will develop metastasis at a younger age. Accordingly, Kaplan-Meier estimates of cumulative metastasis yielded distinct curves, indicating that these events occurred earlier in individuals with the S836S polymorphic allele (Figure 1).

The presence of S836S variant allele was also associated with younger age and a higher percentage of local and distant metastatsis at diagnosis in a sample of sporadic MTC patients (Table 5 and Figure 2). A possible role of this variant in the pathogenesis of sporadic MTC has been speculated by several studies. Similar to the findings of this study, the S836S variant allele was over-represented in sporadic MTC patients from Germany, Spain and the United States (Gimm et al. 1999; Ruiz et al. 2001) but no differences were observed between controls and French, Polish, British, Chilean, Portuguese and Austrian patients (Wiench, et al. 2001; Berard, et al. 2004; Baumgartner-Parzer et al. 2005; Cebrian, et al. 2005; Costa, et al. 2005; Wohllk N 2005). The reasons for these conflicting results are still unclear. Yet, a significantly higher frequency of the S836S variant in patients with M918T somatic mutation in sporadic MTC has been reported (Gimm et al. 1999). Of interest, a kindred where the carriers of S836S developed MTC (Gimm et al. 1999) and a case of C-cell hyperplasia and primary HPT in an individual harboring the S836S polymorphism in the absence of germline *RET* mutations have also been reported (Brauckhoff, et al. 2002).

In contrast to the large number of studies focused on the role of S836S variant in sporadic MTC, we have found only a few focused in hereditary MTC. Tamanaha et al demonstrated that the S836S variant was over-represented in G533C carriers and non-carrier family members compared to control population. However, they were unable to show an association between this variant and earlier age at onset in the large kindred studied (Tamanaha et al. 2009). A modulating effect of the combination polymorphic L769L with wild-type S836S on the clinical outcome of hereditary MTC has also been described (Severskaia et al. 2006). The segregation of *RET* V804L germline mutation and the S836S variant was reported in a Hungarian FMTC kindred comprising 80 individuals of

four generations but the co-existence of the V804L mutation and S836S polymorphism did not seem to aggravate the relatively low-risk disease phenotype (Patocs, et al. 2003).

The exact mechanism by which these polymorphisms modulate MTC pathogenesis or disease presentation is still not known and open to speculation. Even though these allelic variants do not seem to confer any transforming activity on the tyrosine kinase domain of the RET protein, cumulative studies suggest that they might modify disease susceptibility and clinical phenotype in patients with sporadic or hereditary MTC. Because it has been reported that polymorphic sequence variants can lead to the production of different amounts of mRNA, a theoretical role of the polymorphisms in the pathogenesis of MTC is that the allelic variant might influence *RET* mRNA expression. However, Elisei et al did not find any significant difference in the levels of *RET* mRNA when comparing sporadic MTC patients with or without G691S/S904S, L769L or S836S polymorphism (Elisei et al. 2004). The S836S polymorphism failed to affect DNA–protein binding, transcript stability or RNA splicing and editing (Griseri, et al. 2000), but it is possible that this genetic variant may create an unstable sequence upstream or downstream at germline or somatic *RET* mutations instead of directly participating in the tumourigenic process (Gimm et al. 1999). Such a mechanism has been observed in the *APC* gene in a fraction (28%) of Ashkenazim with familial colorectal cancer where additional somatic mutations were more often found on that allele carrying a seemingly innocuous germline missense mutation predicted to result in a conservative amino acid change (Laken, et al. 1997).

A novel observation in this study was the association between the S836S polymorphic allele and early metastatic disease observed in both hereditary and sporadic MTC, suggesting that this variant might interfere in tumor progression. The S836S allele did not co-segregate with the germline mutation and was aleatory distributed among individuals with 634 mutations, the most prevalent mutation in our series. Accordingly, statistical analysis performed using the Cox proportional hazard survival model identified the S836S variant as an independent risk factor for metastatic disease in MTC (hazard ratio 2.82, Table 6). A possible confounding effect of somatic M918T mutation in the sporadic group was also addressed. The M918T mutation was present in about 80% of the samples analyzed, a frequency similar to that reported in some centers (Zedenius, et al. 1995; Marsh, et al. 1996; Moura, et al. 2009), but that could be influenced by selection since most of the available samples were from patients followed at our Institution due to advanced disease. Although we observed a much higher frequency of the S836S variant (35%) in this subgroup of patients, there was no significant association between somatic M918T mutation and the S836S polymorphic allele (P=0.383).

As previously discussed, the mechanistic explanation is speculative. An additional hypothesis is that an unknown functional variant, which may be in linkage disequilibrium with the haplotype containing the S836S variant, could possibly control the activity of *RET* oncogene. A MTC-specific risk haplotype that includes the S836S and IVS1–126G > T *RET* polymorphisms was previously described (Borrego, et al. 2003). The IVS1–126G > T variant has also been associated with the development of sporadic MTC (Fernandez, et al. 2004) and, interestingly, with earlier age at disease onset in a large Brazilian kindred harboring the G533C *RET* mutation (Tamanaha et al. 2009). Of note, an *in silico* analysis revealed that this genetic variant creates a new binding site for NFAT transcription factor (nuclear factor of activated T-cells (Borrego et al. 2003). The NFAT family of proteins has been found to be involved in cell cycle regulation, cell differentiation, cell survival, angiogenesis, tumour cell invasion and metastasis (Lu and Huan 2007).

The *RET* variants L769L and G691S/ S904S have also been studied as modifiers in disease presentation in both hereditary and sporadic MTC patients (Berard et al. 2004; Fernandez et al. 2006a; Fernandez, et al. 2006b; Guerrero, et al. 2006). The polymorphic G691S/ S904S variant of *RET* has been implicated as a modifier factor on the age at which MEN 2A begins (Gil, et al. 2002; Robledo et al. 2003), whereas the *RET* L769L polymorphism has been previously implicated as having an effect in the early development of hereditary MTC in a family with a mutation in exon 14 (Magalhaes et al. 2004). However, we did not observe an association between the presence of these variants and clinical presentation of the disease in hereditary MTC. This highlights the importance of replication in different populations and might indicate differences due to genetic background or geographic areas.

Some factors unrelated to the *RET* polymorphisms could have interfered with the findings of the present study. Firstly, our results could represent a type 1 error. However, the S836S was significantly associated to younger age at diagnosis and early metastatic disease in patients with clinical disease in hereditary and sporadic MTC groups. These results argue against an association by chance. Secondly, due to the relatively small percentage of cases (38%) analyzed, we cannot formally rule out a confounding effect from the somatic M918T mutation in the sporadic group. Finally, this study has insufficient statistical power to exclude the possibility of an association between the L769L or G691S/S904S variants and clinical presentation of the disease in hereditary MTC (type 2 error).

In conclusion, our data indicate that the *RET* variant S836S is associated with increased risk for metastatic disease at a younger age in individuals with MEN 2A or sporadic MTC. If confirmed in other sample populations, these findings might have significant implications on the management of MTC, particularly on defining the ideal timing for prophylactic intervention on gene carriers.

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Disclosure statement

There are no competing financial interests.

References:

Baumgartner-Parzer SM, Lang R, Wagner L, Heinze G, Niederle B, Kaserer K, Waldhausl W & Vierhapper H 2005 Polymorphisms in exon 13 and intron 14 of the RET protooncogene: genetic modifiers of medullary thyroid carcinoma? *J Clin Endocrinol Metab* **90** 6232-6236.

Berard I, Kraimps JL, Savagner F, Murat A, Renaudin K, Nicolli-Sire P, Bertrand G, Moisan JP & Bezieau S 2004 Germline-sequence variants S836S and L769L in the RE arranged during Transfection (RET) proto-oncogene are not associated with predisposition to sporadic medullary carcinoma in the French population. *Clin Genet* **65** 150-152.

Borrego S, Wright FA, Fernandez RM, Williams N, Lopez-Alonso M, Davuluri R, Antinolo G & Eng C 2003 A founding locus within the RET proto-oncogene may account for a large proportion of apparently sporadic Hirschsprung disease and a subset of cases of sporadic medullary thyroid carcinoma. *Am J Hum Genet* **72** 88-100.

Brauckhoff M, Gimm O, Bilkenroth U, Hinze R & Dralle H 2002 Primary hyperparathyroidism, C-cell hyperplasia and papillary thyroid carcinoma in a patient with RET germline polymorphism S836S. *Langenbecks Arch Surg* **387** 201-203.

Cebrian A, Lesueur F, Martin S, Leyland J, Ahmed S, Luccarini C, Smith PL, Luben R, Whittaker J, Pharoah PD, et al. 2005 Polymorphisms in the initiators of RET (rearranged during transfection) signaling pathway and susceptibility to sporadic medullary thyroid carcinoma. *J Clin Endocrinol Metab* **90** 6268-6274.

Costa P, Domingues R, Sobrinho LG & Bugalho MJ 2005 RET polymorphisms and sporadic medullary thyroid carcinoma in a Portuguese population. *Endocrine* **27** 239-243.

Elisei R, Cosci B, Romei C, Bottici V, Renzini G, Molinaro E, Agate L, Vivaldi A, Faviana P, Basolo F, et al. 2008 Prognostic significance of somatic RET oncogene mutations in sporadic medullary thyroid cancer: a 10-year follow-up study. *J Clin Endocrinol Metab* **93** 682-687.

Elisei R, Cosci B, Romei C, Bottici V, Sculli M, Lari R, Barale R, Pacini F & Pinchera A 2004 RET exon 11 (G691S) polymorphism is significantly more frequent in sporadic medullary thyroid carcinoma than in the general population. *J Clin Endocrinol Metab* **89** 3579-3584.

Eng C, Clayton D, Schuffenecker I, Lenoir G, Cote G, Gagel RF, van Amstel HK, Lips CJ, Nishisho I, Takai SI, et al. 1996 The relationship between specific RET proto-oncogene mutations and disease phenotype in multiple endocrine neoplasia type 2. International RET mutation consortium analysis. *JAMA* **276** 1575-1579.

Fernandez RM, Navarro E, Antinolo G, Ruiz-Ferrer M & Borrego S 2006a Evaluation of the role of RET polymorphisms/haplotypes as modifier loci for MEN 2, and analysis of the correlation with the type of RET mutation in a series of Spanish patients. *Int J Mol Med* **17** 575-581.

Fernandez RM, Pecina A, Antinolo G, Navarro E & Borrego S 2006b Analysis of RET polymorphisms and haplotypes in the context of sporadic medullary thyroid carcinoma. *Thyroid* **16** 411-417.

Fernandez RM, Robledo M, Antinolo G, Pecina A, Ruiz-Llorente S, Eng C & Borrego S 2004 The RET IVS1-126G>T variant is strongly associated with the development of sporadic medullary thyroid cancer. *Thyroid* **14** 329-331.

Gil L, Azanedo M, Pollan M, Cristobal E, Arribas B, Garcia-Albert L, Garcia-Saiz A, Maestro ML, Torres A, Menarguez J, et al. 2002 Genetic analysis of RET, GFR alpha 1 and GDNF genes in Spanish families with multiple endocrine neoplasia type 2A. *Int J Cancer* **99** 299-304.

Gimm O, Neuberg DS, Marsh DJ, Dahia PL, Hoang-Vu C, Raue F, Hinze R, Dralle H & Eng C 1999 Over-representation of a germline RET sequence variant in patients with sporadic medullary thyroid carcinoma and somatic RET codon 918 mutation. *Oncogene* **18** 1369-1373.

Griseri P, Sancandi M, Patrone G, Bocciardi R, Hofstra R, Ravazzolo R, Devoto M, Romeo G & Ceccherini I 2000 A single-nucleotide polymorphic variant of the RET proto-oncogene is underrepresented in sporadic Hirschsprung disease. *Eur J Hum Genet* **8** 721-724.

Guerrero IM, Pessoa CH, Olmedo DB, Pontes ER, Matos LC, Tilli TM, Barcinski MA & Gimba ER 2006 Analysis of inherited genetic variants in ret proto-oncogene of Brazilian patients with apparently sporadic medullary thyroid carcinoma. *Thyroid* **16** 9-15.

Kouvaraki MA, Shapiro SE, Perrier ND, Cote GJ, Gagel RF, Hoff AO, Sherman SI, Lee JE & Evans DB 2005 RET proto-oncogene: a review and update of genotype-phenotype correlations in hereditary medullary thyroid cancer and associated endocrine tumors. *Thyroid* **15** 531-544.

Laken SJ, Petersen GM, Gruber SB, Oddoux C, Ostrer H, Giardiello FM, Hamilton SR, Hampel H, Markowitz A, Klimstra D, et al. 1997 Familial colorectal cancer in Ashkenazim due to a hypermutable tract in APC. *Nat Genet* **17** 79-83.

Lesueur F, Cebrian A, Robledo M, Niccoli-Sire P, Svensson KA, Pinson S, Leyland J, Whittaker J, Pharoah PD & Ponder BA 2006 Polymorphisms in RET and its coreceptors and ligands as genetic modifiers of multiple endocrine neoplasia type 2A. *Cancer Res* **66** 1177-1180.

Lu H & Huan C 2007 Transcription factor NFAT, its role in cancer development, and as a potential target for chemoprevention. *Curr Cancer Drug Targets* **7** 343-353.

Machens A, Gimm O, Hinze R, Hoppner W, Boehm BO & Dralle H 2001 Genotype-phenotype correlations in hereditary medullary thyroid carcinoma: oncological features and biochemical properties. *J Clin Endocrinol Metab* **86** 1104-1109.

Machens A, Niccoli-Sire P, Hoegel J, Frank-Raue K, van Vroonhoven TJ, Roeher HD, Wahl RA, Lamesch P, Raue F, Conte-Devolx B, et al. 2003 Early malignant progression of hereditary medullary thyroid cancer. *N Engl J Med* **349** 1517-1525.

Magalhaes PK, de Castro M, Elias LL, Soares EG & Maciel LM 2004 Polymorphisms in the RET proto-oncogene and the phenotypic presentation of familial medullary thyroid carcinoma. *Thyroid* **14** 848-852.

Marsh DJ, Learoyd DL, Andrew SD, Krishnan L, Pojer R, Richardson AL, Delbridge L, Eng C & Robinson BG 1996 Somatic mutations in the RET proto-oncogene in sporadic medullary thyroid carcinoma. *Clin Endocrinol (Oxf)* **44** 249-257.

Milos IN, Frank-Raue K, Wohllk N, Maia AL, Pusiol E, Patocs A, Robledo M, Biarnes J, Barontini M, Links TP, et al. 2008 Age-related neoplastic risk profiles and penetrance estimations in multiple endocrine neoplasia type 2A caused by germ line RET Cys634Trp (TGC>TGG) mutation. *Endocr Relat Cancer* **15** 1035-1041.

Moura MM, Cavaco BM, Pinto AE, Domingues R, Santos JR, Cid MO, Bugalho MJ & Leite V 2009 Correlation of RET somatic mutations with clinicopathological features in sporadic medullary thyroid carcinomas. *Br J Cancer* **100** 1777-1783.

O'Sullivan B & Shah J 2003 New TNM staging criteria for head and neck tumors. *Semin Surg Oncol* **21** 30-42.

Patocs A, Valkusz Z, Igaz P, Balogh K, Toth M, Varga I & Racz K 2003 Segregation of the V804L mutation and S836S polymorphism of exon 14 of the RET gene in an extended kindred with familial medullary thyroid cancer. *Clin Genet* **63** 219-223.

Ponder BA 1999 The phenotypes associated with ret mutations in the multiple endocrine neoplasia type 2 syndrome. *Cancer Res* **59** 1736s-1741s; discussion 1742s.

Punales MK, da Rocha AP, Meotti C, Gross JL & Maia AL 2008 Clinical and oncological features of children and young adults with multiple endocrine neoplasia type 2A. *Thyroid* **18** 1261-1268.

Punales MK, Graf H, Gross JL & Maia AL 2003 RET codon 634 mutations in multiple endocrine neoplasia type 2: variable clinical features and clinical outcome. *J Clin Endocrinol Metab* **88** 2644-2649.

Robledo M, Gil L, Pollan M, Cebrian A, Ruiz S, Azanedo M, Benitez J, Menarguez J & Rojas JM 2003 Polymorphisms G691S/S904S of RET as genetic modifiers of MEN 2A. *Cancer Res* **63** 1814-1817.

Rocha AP, Magalhaes PK, Maia AL & Maciel LM 2007 [Genetic polymorphisms: implications in the pathogenesis of medullary thyroid carcinoma]. *Arq Bras Endocrinol Metabol* **51** 723-730.

Ruiz A, Antinolo G, Fernandez RM, Eng C, Marcos I & Borrego S 2001 Germline sequence variant S836S in the RET proto-oncogene is associated with low level predisposition to sporadic medullary thyroid carcinoma in the Spanish population. *Clin Endocrinol (Oxf)* **55** 399-402.

Severskaia NV, Saenko VA, Il'in AA, Chebotareva IV, Rumiantsev PO, Isaev PA, Medvedev VS & Iasmita S 2006 [RET and GFRA1 germline polymorphisms in medullary thyroid cancer patients]. *Mol Biol (Mosk)* **40** 425-435.

Tamanaha R, Camacho CP, Pereira AC, da Silva AM, Maciel RM & Cerutti JM 2009 Evaluation of RET polymorphisms in a six-generation family with G533C RET mutation: specific RET variants may modulate age at onset and clinical presentation. *Clin Endocrinol (Oxf)* **71** 56-64.

Wiench M, Wloch J, Wygoda Z, Gubala E, Oczko M, Pawlaczek A, Kula D, Lange D & Jarzab B 2004 RET polymorphisms in codons 769 and 836 are not associated with predisposition to medullary thyroid carcinoma. *Cancer Detect Prev* **28** 231-236.

Wiench M, Wygoda Z, Gubala E, Wloch J, Lisowska K, Krassowski J, Scieglinska D, Fiszer-Kierzkowska A, Lange D, Kula D, et al. 2001 Estimation of risk of inherited medullary thyroid carcinoma in apparent sporadic patients. *J Clin Oncol* **19** 1374-1380.

Wohllk N SE, Bravo M, Becker P 2005 Polimorfismos G691S, L769L y S836S del proto-oncogene RET no se associan a mayor riesgo de cancer medular tiroideo esporádico en pacientes chilenos. *Rev Med Chil* **133** 397-402

Zedenius J, Larsson C, Bergholm U, Bovee J, Svensson A, Hallengren B, Grimelius L, Backdahl M, Weber G & Wallin G 1995 Mutations of codon 918 in the RET proto-oncogene correlate to poor prognosis in sporadic medullary thyroid carcinomas. *J Clin Endocrinol Metab* **80** 3088-3090.

Parte III

Molecular Signaling Pathways and Targeted Therapy in Medullary Thyroid Carcinoma

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MOLECULAR SIGNALING PATHWAYS AND TARGETED THERAPY IN MEDULLARY THYROID CARCINOMA

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Running title: Medullary thyroid carcinoma, signaling pathways and tyrosine-kinase inhibitors

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Abstract

Medullary thyroid carcinoma (MTC) accounts for 5-10% of all thyroid malignancies. Activating mutations of the *RET* protooncogene are invoveld in the pathogenesis of MTC. Receptor tyrosine kinases (RTKs) play an important role in cell proliferation and survival through the activation of downstream signaling pathways, including MAPK and PI3K pathways. The molecules involved in the tumorigenesis process serve as potential therapeutic targets for new drugs, currently under study in the management of MTC. These novel agents are an attractive strategy, because conventional treatments have limited response rates in metastatic disease. The most studied drugs are tyrosine kinase inhibitors, such as sorafenib, sunitinib and vandetanib. Studies with these molecules have shown promising results with 40% and 53% of partial response and stable disease rate, respectively. However, it is important to remember that these medications have a high cost, as well as a profile of adverse events that may limit their use in clinical practice.

INTRODUCTION

Medullary thyroid carcinoma (MTC), a malignant neoplasia of the parafollicular C cells of the thyroid, accounts for 5-10% of all thyroid malignancies. MTC may occur sporadically, approximately 75% of the cases or as part of the inherited cancer syndrome of multiple endocrine neoplasia type 2 (MEN 2). The hereditary form of MTC is associated with germline mutations in the *RET* (*RE arranged during Transfection*) proto-oncogene. This hereditary syndrome includes three clinically distinct forms: MEN 2A, MEN 2B, and familial MTC (FMTC). In patients with FMTC, only the thyroid is affected. Patients with MEN 2A develop MTC, pheochromocytoma (PHEO), and/or primary hyperparathyroidism (HPT). MEN 2B patients have MTC, PHEO, ganglioneuromas of the digestive tract, mucosal neuromas, and/or skeletal abnormalities (Ceolin, et al. 2012).

Cervical lymphadenopathy is an early manifestation in the clinical course of MTC and 50-75% of the patients had cervical lymph node metastasis at diagnosis (Moley and DeBenedetti 1999; Scollo, et al. 2003). Distant metastases are detected in 7-17% of MTC patients at diagnosis and frequently involve multiple sites (lung, liver, bones and more rarely, brain and skin) (Bergholm, et al. 1989; Giraudet, et al. 2007; Girelli, et al. 1998; Kebebew, et al. 2000). Despite the high frequency of metastatic disease at diagnosis, tumor growth is usually slow in MTC.

A large study of an American cohort of MTC patients demonstrated that age at diagnosis, stage of disease and extent of surgery are important predictors of survival. Patients with tumor confined to the thyroid gland had a 10-year survival rate of 95.6%, whereas patients with regional stage disease or distant metastasis at diagnosis had an overall survival rate of 75.5% and 40%, respectively (Roman, et al. 2006).

Surgery is the only curative treatment for MTC (Kebebew et al. 2000; Pelizzo, et al. 2007; Scollo et al. 2003). There are no effective therapeutic options for distant metastatic disease, since chemotherapy and external beam radiation therapy for metastatic or cervical recurrent disease have limited response rates (Brierley, et al. 1996; Nocera, et al. 2000).

The main challenge in the management of MTC is patients with advanced and progressive disease, because the current therapeutic options have poor results for these individuals. In the last few years, several studies have improved knowledge about the molecular biology of MTC. Tyrosine kinase receptors, such as RET and vascular endothelial growth factor-A (VEGFA) involved in proliferation and cell survival, play an important role in the tumorigenesis process. In response to binding of extracellular ligands, such receptors are phosphorylated and activated downstream signaling pathways, such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-

kinase (PI3K) pathways and many signalling effectors, like β-catenin and nuclear factor- kappa B (NF-κB) (Ichihara, et al. 2004; Vieira, et al. 2005). The molecules involved in these processes serve as potential therapeutic targets for new drugs (Broekman, et al. 2011).

In the present study, we review the main pathways involved in the development of MTC and the potential targeted therapies for patients with persistent or recurrent disease.

TYROSINE KINASE RECEPTORS

RET receptor

The *RET* protooncogene is the susceptibility gene for hereditary MTC (Donis-Keller, et al. 1993; Ishizaka, et al. 1989; Mulligan, et al. 1993). This protooncogene encodes a receptor tyrosine kinase (RTK) expressed primarily in neural crest and urogenital precursor cells. The RET protein consists of an extracellular region (four cadherin-like domains and a cysteine-rich domain), a transmembrane domain, and two intracellular tyrosine kinase domains).The RET receptor plays a role in regulating cell proliferation, migration, differentiation and survival. In the absence of ligand, this RTK is monomeric, unphosphorylated and hence inactive. Binding of the ligand to the extracellular domain induces receptor dimerization and autophosphorylation (de Groot, et al. 2006a).

RET is activated through the binding of four Glial cell line-derived neurotrophic factor (GDNF) family ligands neurturin, artemin, persephin and GDNF together with the four corresponding membrane co-receptors of GDNF family receptor α (GFRα 1,2,3 and 4) (de Groot et al. 2006a).

Activation of RET results in phosphorylation of its intracellular tyrosine residues, which act as docking sites for various adaptor proteins; for example, the tyrosine Y981, Y1015 and Y1062 represent binding sites for Src, phospholipase C gamma (PLCγ) and Shc, respectively. After these bindings various signaling pathways are activated, such as PI3K, MAPK-ERK/p38 and c-Jun N-terminal kinase (JNK) pathways. Intriguingly, all of these pathways are activated mainly through Y1062 of RET (Airaksinen and Saarma 2002; Ichihara et al. 2004). Accordingly, a previous study demonstrated *in vivo* that the replacement of Y1062 with phenylalanine (Y1062F mutation) severely impaired the transforming activity of MEN2A-RET and MEN2B-RET, indicating that Y1062 represents a major binding site for the Shc adaptor proteins- signaling molecules responsible for cell transformation (Figure 1) (Asai, et al. 1996).

Src family kinases- nonreceptor TKs - are implicated in signal transduction mediated by transmembrane receptors. Src activation is essential for the mitogenic activity of RET. Interestingly, when the RET receptor is activated by mutations, the cells showed higher Src kinase activity than normal (Melillo, et al. 1999).

MTC-associated *RET*-mutations convert *RET* into a dominantly transforming oncogene. Activating mutations of *RET* have been reported in nearly all hereditary cases of MTC and in 50-80% of sporadic tumor cases (Elisei, et al. 2008; Kouvaraki, et al. 2005; Marsh, et al. 1996; Moura, et al. 2009; Siqueira, et al. 2010). In sporadic MTC the presence of a somatic *RET* mutation correlates with a worse outcome for the highest probability to have persistent disease and a lower survival rate (Elisei et al. 2008; Schilling, et al. 2001). The germline *RET* mutations are described mainly in exons 10, 11 and 16, thus can involve the extracellular or intracellular domains. The most frequent mutations are located in codon 634 in the extracellular cysteine-rich domain, occurring in more than 60% of all genetically identified MTC. (Ceolin et al. 2012) Extracellular cysteine *RET*-mutants exert constitutive kinase activity consequent to ligand-independent homodimerization. Mutations in the tyrosine kinase (TK) domain lead to constitutive RET activation probably resulting from disruption of an auto-inhibited head-to-tail RET TK homodimer (Borrello, et al. 1995; Santoro, et al. 1995). There are important differences with regard to activation of intracellular signaling among these different mutations that might partially explain the genotype-phenotype correlation of the hereditary MTC (Iwashita, et al. 1996). For example, Puñales et al. observed in MEN 2 patients that the genotype C634R presented significantly more distant metastases at diagnosis than groups C634W and C634Y, thus suggesting that a change of specific amino acids may modify the natural development of the disease (Punales, et al. 2003). It is interesting to note that no mutation within the coding regions of the *GDNF* gene has been reported in MTC (Borrego, et al. 1998; Marsh, et al. 1997).

VEGF receptor

VEGF-A plays a key role in angiogenesis, an essential step in tumor growth; this molecule stimulates proliferation, migration, and survival of endothelial cells (Vieira et al. 2005). VEGF mediates its biological effects by binding RTKs, VEGF receptor 1 (VEGFR-1/Flt-1) and VEGF receptor 2 (VEGFR-2/Flk-1/KDR) (Shibuya and Claesson-Welsh 2006). The VEGF-A, VEGFR-1, and VEGFR-2 have been demonstrated to be overexpressed in MTC lesions and might be implicated in tumor progression (Capp, et al. 2010; de la Torre, et al. 2006). These findings demonstrate a biological basis for therapy with inhibitors of tyrosine kinase receptors (TKI).

EGF receptor

The epidermal growth factor receptor (EGFR) has an important role in cell proliferation, survival, differentiation, migration and angiogenesis (Normanno, et al. 2006). In tissues samples of MTC, EGFR was expressed frequently and strongly within the cytoplasm; this factor could be important for tumor cell proliferation (Wang, et al. 1997). Besides the EGFR, its ligands (EGF and TGFα) are also expressed by C-cells and MTC (van der Laan, et al. 1995). However, no EGFR-activating TK domain mutations were found in 9 MTC specimens (Mitsiades, et al. 2006a). Interestingly, in cell lines carrying *RET/PTC* rearrangements, there is evidence that EGFR contributes to RET kinase activation, signaling, and growth stimulation (Croyle, et al. 2008). A study in both the medullary carcinoma cell lines (TT and MZ-CRC-1) observed that the phosphorylated EGFR became highly expressed after EGF stimulation. (Gorla, et al. 2009).

MET receptor

The hepatocyte growth factor (HGF) receptor is called MET and it has a transmembrane tyrosine kinase domain (Bottaro, et al. 1991). HGF, a single-chain inert precursor converted into a two-chain functional heterodimer by extracellular proteases, is a mesenchyme-derived cytokine that is produced in the early phases of the regenerative processes that act as a mitogen of many epithelial cells (Trusolino, et al. 2010). Following HGF binding, the kinase activity of MET leads to activation of downstream signal transduction pathways that include the MAPK and the PI3K cascades, signal transducer and activator of transcription proteins (STATs), and NF-κB complex; all of these pathways regulate cell proliferation, survival and migration (Trusolino et al. 2010).

The HGF-MET system appears to be involved in MTC. Expression of the MET receptor (confined to the cell membrane) and HGF (in the cell membrane and intracellular region) has been demonstrated in a limited number of MTC cells. (Trovato, et al. 1998). Analysis of the *MET* mutations in MTC tissue found missense MET sequence in 8% of the cases, but the clinical and the molecular pathologic significance of this MET sequence alteration is unknown (Wasenius, et al. 2005).

HGF/MET signaling contributes to oncogenesis and tumor progression and promotes aggressive cellular invasiveness that is strongly linked to tumor metastasis, thus drugs that inhibit this pathway become an interesting therapeutic option (Cecchi, et al. 2010).

INTRACELULAR SIGNALING PATHWAYS

After activation, RET transmits signals to promote proliferation, motility and cell survival (Asai, et al. 2006; Kodama, et al. 2005). The main signaling pathways activated by RET are MAPK (ERK/p38) and PI3K cascades. RAS and PI3K contribute to the activation of many signaling effectors including NF-κB, STAT and β-catenin (Cerrato, et al. 2009).

MAPK pathway

The MAPK cascades are central signaling pathways that regulate different cellular processes, including proliferation, differentiation and apoptosis. The dysregulation of these cascades is involved in the induction and progression of cancer. Four mammalian MAPK cascades have been identified and named according to their MAPK components: extracellular signal-regulated kinase 1 and 2 (ERK1/2), JNK, p38, and ERK5. Constitutive activation of MAPK in cancer occurs through activating mutations or overexpression of upstream effectors in the pathway, primarily of genes encoding receptor tyrosine kinases, RAS and RAF (Knauf and Fagin 2009; Plotnikov, et al. 2011).

The MAPK (ERK/p38) cascade is best characterized by the RAS-effector pathway. RAS proteins are small GTPases that cycle between inactive guanosine diphosphate (GDP)-bound and active guanosine triphosphate (GTP)-bound conformations. Growth-factor binding to cell surface RTKs creates docking sites for adaptor molecules that recruit and activate guanine nucleotide-exchange factors (GNEFs), which in turn favors GTP binding to RAS small G-proteins (KRAS, HRAS and NRAS) (Schubbert, et al. 2007). Once activated, RAS stimulates effector molecules, including RAF, PI3K and RAL guanine nucleotide-dissociation stimulator, to regulate proliferation, survival and differentiation (Halilovic and Solit 2008). There are three RAF serine/threonine kinases (ARAF, BRAF and CRAF) that activate the MEK- ERK kinase cascade (Figure 1) (Schubbert et al. 2007).

The RAS pathway can be triggered by point mutations in the *RAS* genes, by loss of the negative regulator neurofibromin (NF1), by upstream activation of cell surface RTKs or by downstream activation of RAS signaling effectors (Halilovic and Solit 2008).

The analysis of tumor tissue of 25 patients with *RET*-negative sporadic MTC identified somatic *HRAS* and *KRAS* mutations in 56% and 12% of the tumor, respectively; but no *BRAF* or *NRAS* somatic mutation was found in this population (Moura, et al. 2011). Another study in a Greek cohort described the presence of *KRAS* and *BRAF* mutations in 41% and 68% of MTC samples

analyzed, respectively (Goutas, et al. 2008). However, previous studies did not identify mutations in members of the RAS-family or RAF-family in samples of MTC (Bockhorn, et al. 2000; Moley, et al. 1991).

PI3K pathway

Class-I phosphatidylinositol-3 kinases (PI3Ks) are heterodimers composed of a catalytic subunit (p110) and an adaptor/regulatory subunit (p85). This class is activated by RTK or receptors coupled with G proteins (Fresno Vara, et al. 2004). PI3Ks constitute a lipid kinase family characterized by their ability to phosphorylate the inositol ring 3'-OH group in inositol phospholipids to generate the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3). Then PIP3 activates downstream molecules such as the RAC small GTPase, 3-phosphoinositide-dependent protein kinase 1 (PDPK1) and the AKT serine/threonine kinase (Yuan and Cantley 2008). AKT stimulates the serine/threonine kinase mammalian target of rapamycin (mTOR), this in turn contributes to NF-κB activation (Bjornsti and Houghton 2004). The PI3K/AKT/mTOR cascade promotes growth, cell proliferation and prevents cell death (Fresno Vara et al. 2004). PI3K activation may occur independently of the receptor, as in the case of cells expressing constitutively active RAS (Kauffmann-Zeh, et al. 1997). In PI3K pathway, phosphatase and tensin homolog (PTEN) acts as a negative regulator of activation (Figure 1). *PTEN* gene is frequently mutated in advanced stages of several human tumors (Fresno Vara et al. 2004).

Higher levels of phosphorylated AKT protein were found in the MTC cell line (TT cells), suggesting that the PI3K/AKT pathway is active. Moreover, specific inhibition of this pathway by LY294002 decreases cell proliferation due to induction of apoptosis. Therefore, strategies to inactivate AKT may be a therapeutic alternative in the MTC management (Kunnimalaiyaan, et al. 2006).

Although, mutations of catalytic subunit p110α of *PI3K* gene (*PIK3CA*) have not been found in MTC samples (Wu, et al. 2005), there is evidence that the prolonged activation of the PI3K/AKT pathway is a key event that accounts for the oncogenic ability of the MEN2A form of RET (Segouffin-Cariou and Billaud 2000). Thus the PI3K/AKT pathway may play a role in MTC.

NF-κB signaling effector

The NF-κB family of transcription factors controls various cellular processes - cell proliferation, angiogenesis, metastasis and inflammation. The NF-κB family includes five

transcription factors named NF-κB 1 (p50), NF-κB 2 (p52), REL, RELA (p65) and RELB. NF-κB proteins are inactive through binding to inhibitors, known as the IKB proteins (α,β,ε). The activation of NF-κB depends on phosphorylation –induced ubiquitination of IKB mediated by the IKB kinase (IKK) complex (Figure 1) (Baud and Karin 2009).

In the MTC, RET stimulates IKK phosphorylation (Encinas, et al. 2008). NF-κB has recently been shown to play an important role in thyroid cancer for its ability to control the proliferative and the anti-apoptotic signaling pathways of thyroid neoplastic cells (Pacifico and Leonardi 2010; Palona, et al. 2006; Visconti, et al. 1997).

A previous study in a MTC cell line (TT cells) showed that NF-κB represents a downstream target of oncogenic RET; their result demonstrated that RET-induced NF-κB activation depends on IKK-mediated IKBα degradation and requires functional RAS, RAF, and MEKK1. However, RET induced NF-κB activation was not accomplished by MEK/ERK proteins belonging to the classical mitogenic kinase cascade or by PI3K/AKT, which are known to be involved in NF-kB activation (Ludwig, et al. 2001). More recently, the presence of members of NF-κB was described, for example p65 and p52, in the nucleus of hereditary or sporadic MTC cells. Interestingly, MTC with germ line or somatic *RET* mutations showed NF-κB nuclear translocation of p65 more frequently than MTC without *RET* mutation, suggesting that RET activation by somatic or germ line mutations may be responsible for NF-κB activation in MTC (Gallel, et al. 2008).

A number of specific or non-specific NF-κB inhibitors have been tried to take over the cascade in *in vitro* and *in vivo* experiments. These agents can induce massive apoptosis especially in combination with radio- or chemotherapy. Current results suggest that the inhibition of NF-κB may be a promising strategy for advanced thyroid cancer. Nevertheless, further studies are still required to develop specific and clinically effective NF-κB inhibitors (Namba, et al. 2007).

NF-κB inhibitory molecules have been demonstrated to promote thyroid cell death alone or in combination with chemo-therapy or radiation exposure (Mitsiades, et al. 2006b; Starenki, et al. 2004). The proteosome inhibitor bortezomib (PS-341, Velcade), which is the only pharmacological inhibitor of NF-κB approved by FDA for the treatment of multiple myeloma , induced apoptosis in medullary and anaplastic cell lines with IC(50) values well within the range of clinically achievable concentrations (Mitsiades et al. 2006b).

β-catenin pathway

β-catenin, encoded by the *CTNNB1* gene, is a multifunctional protein that plays important roles in signal transduction and cellular adhesion by associating with E-cadherin and α-catenin. At

the plasma membrane, β-catenin is associated with E-cadherin and α-catenin in linking the cytoskeleton and adherens junctions, whereas in the nucleus, it acts as a transcription factor, through the TCF/LEF (T-cell factor/lymphoid-enhancing factor), regulating expression of genes involved in cell migration and survival (Brembeck, et al. 2006; Harris and Peifer 2005; Morin 1999). It has been demonstrated that phosphorylation of β -catenin by the serine-threonine kinase GSK3- β is crucial for destabilization of β –catenin (Morin 1999).

In papillary thyroid carcinoma, RET/PTC oncoproteins stimulate β-catenin activation via direct phosphorylation on Y654 and via PI3k/AKT and RAS/ERK (Cassinelli, et al. 2009; Castellone, et al. 2009). β-catenin, E-cadherin and occludin are presented at high levels in human MTC cells (TT cell line). However, the activation of the MAPK (ERK/p38) pathway markedly decreased the expression of these essential components of cell–cell contacts. This RAF-1–induced reduction in the expression of E-cadherin, β-catenin, and occludin suggest that the MAPK (ERK/p38) pathway plays an important role in the regulation of cell–cell contacts in MTC cells (Ning, et al. 2008).

In human MTC samples, nuclear β-expression was evidenced in 8 of 20 patients with oncogenic RET, yet these nuclear localization of β-catenin was not detected in normal or hyperplastic C cells in human tissues. Besides that, nuclear localization was more prevalent in metastasis than in primary tumor. These data suggest an association between aggressive MTC and activation of β-catenin. It was also demonstrated that RET can directly phosphorylates β-catenin and that the tyrosine phosphorylation of β-catenin is not dependent on SRC activation.(Gujral, et al. 2008).

STAT pathway

STAT transcription factors are activated in response to cytokines, through janus kinase (JAK), or directly by RTK (Schindler and Darnell 1995; Stahl, et al. 1995). In normal cells, STAT3 is mainly found in cytoplasm and it is not phosphorylated, but in neoplasic cells the phosphorylated STAT has a nuclear localization (Plaza Menacho, et al. 2005). It has previously been shown that the RET receptor contains two STAT3-specific docking sites (Y752 and Y928) and oncogenic activation of STAT3 by RET C634R is mediated by the intrinsic tyrosine kinase domain of RET, independently of JAKs and Src (Schuringa, et al. 2001). However, in *RET* Y791F and *RET* S891A mutants, STAT3 activation is mediated by a signaling pathway involving Src and JAK (Plaza Menacho et al. 2005). These findings suggest that different *RET* mutations activate STAT3 through different signaling routes (Figure 1).

The JAK/STAT pathway is also involved in the process of cell growth arrest in MTC cells induced by leukemia inhibitory factor (LIF) (Arthan, et al. 2010; Auernhammer and Melmed 2000). Interestingly, in MTC cells harboring a *RET* mutation, the activation of the MAPK pathway can induce growth arrest by secreting LIF (Arthan et al. 2010; Hong, et al. 2009; Park, et al. 2003). Although aberrant activation of RAS or its downstream effector RAF had been described in carcinogenesis processes, the sustained activation of these molecules induces growth arrest in normal cells (Braig and Schmitt 2006). These results suggest the possibility of a new therapeutic target in MTC (Arthan et al. 2010).

TUMOR SUPPRESSOR GENES

TP53 gene

The inactivation of the *TP53* tumor suppressor gene has been observed in a variety of human malignancies (Hahn and Weinberg 2002). Mutations in the *p53* locus have been found in a higher incidence in MTC (53% of the samples harbored single point mutation or LOH of *TP53* gene) (Pavelic, et al. 2006). Moreover, a mutational genotyping analysis for a panel of tumor suppressor genes in MTC demonstrated 44% of allelic loss in *TP53* gene. The frequency of allelic loss was associated with tumoral reccurence and metastasis, it suggesting that this analysis can have prognostic value for MTC (Sheikh, et al. 2004).

Interestingly, experimental studies with Rb-deficient mice (retinoblastoma susceptibility gene) showed that inactivation of p53 further increased the risk of MTC development (Harvey, et al. 1995). However, in a previous study neither *TP53* gene mutations nor positive p53 staining was found in 22 MTC samples (Herfarth, et al. 1997). The findings of the absence or minimal immunohistochemical expression of p53 in MTC have been reproduced by others authors (Basolo, et al. 1997; Wang, et al. 1998).

CDKN2C (p18) and *CDKN1B* (p27) genes

Another two tumor suppressor genes, cyclin-dependent kinase inhibitors *p18 (CDKN2C)* and *p27 (CDKN1B)*, were studied in the MTC tumorigenesis. Both proteins interfere in the cell cycle inhibiting the formation of active cyclin-dependent kinase (CDK) complexes and thus changing the G1-S phase transition of the cell cycle (Sherr and Roberts 1999).

The development of MTC was evaluated in RET2B transgenic mice crossed with p*18* and/or *p27* knockout mice; the results showed that RET2B;*p18*+/- mice and RET2B;*p18*-/- mice developed MTC with a highly increased incidence compared with their corresponding single mutants strains. This indicates that loss of *p18* in combination with oncogenic *RET* increases the risk for MTC development. The heterozygous loss of *p27* resulted in increase of the MTC incidence in RET2B*;p18*+/- or RET2B;*p18*-/- mice, but not in RET2B mice only (van Veelen, et al. 2008). In agreement with these findings, there is evidence that oncogenic *RET* downregulates p18 expression (Joshi, et al. 2007) .

Recently, somatic inactivating *p18* mutations were detected in sporadic and hereditary MTCs, which inhibited p18 function and reduced its stability (van Veelen, et al. 2009).

TARGETING SIGNALING KINASES FOR TREATMENT OF MEDULLARY THYROID CARCINOMA

The cumulative knowledge on the distinct signaling pathways and multiple genetic abnormalities involved in the pathogenesis of cancer has allowed the development of targeted molecular therapies. The protein kinases regulate the processes of cell proliferation, differentiation, migration and anti-apoptotic signaling. One of the most important protein kinases is tyrosine kinase; they are classified in two different groups: receptor tyrosine kinases and cellular tyrosine kinases. These protein kinases are characterized by their ability to catalyze the phosphorylation of tyrosine amino acid residues in proteins and thus activate various intracellular signaling pathways. As already reviewed in this article, the activation of RTK by mutations or over-expression, plays a key role in the development of MTC. Therefore, TKIs serve as therapy for cancer by blocking the tyrosine-kinase –dependent oncogenic pathways. TKIs can be specific to one or several tyrosine kinases - most are designed to inhibit multiple signaling pathways (Broekman et al. 2011; Schlessinger 2000).

The most studied TKI drugs are motesanib, sorafenib, sunitinib, axitinib, imatinib, vandetanib and XL184 (Table 1). Clinical trials with these drugs in patients with MTC have shown promising therapeutic results. In the clinical trials, an objective index called RECIST (Response Evaluation Criteria in Solid Tumors) has been used to evaluate the tumor response. According to RECIST, the tumor lesions are categorized as measurable or nonmeasurable. All measurable lesions up to a maximum of 5 lesions per organ and 10 lesions in total, representative of all involved organs, should be identified as target lesions and recorded and measured at baseline. At the end of

the clinical trial, the responses will be classified as follows: complete response (the disappearance of all target lesions), partial response (at least a 30% decrease in the sum of the longest diameter of target lesions), progressive disease (at least a 20% increase in the sum of the longest diameter of target lesions and stable disease (neither sufficient shrinkage to qualify for partial response nor sufficient increase to qualify for progressive disease) (Therasse, et al. 2000).

Interestingly, the reduction in serum levels of tumor markers (calcitonin and carcinoembryonic antigen) observed with these medications occurs independently of radiological response (Lam, et al. 2010; Licitra, et al. 2010). Another relevant question concerns the different responses of the parenchymal target lesions (for example, metastasis to lung, liver, bone) *vs* nonparenchymal target lesions (metastasis in lymph nodes); one possible explanation is that the parenchymal lesions are better perfused (Machens and Dralle 2010).

Vandetanib (ZD6474, Zactima)

Vandetanib is an agent that selectively targets RET, VEGF and EGF receptors (Carlomagno, et al. 2002; Wedge, et al. 2002). In human MTC cell lines, vandetanib inhibited the cell proliferation and phosphorylation of RET receptor, EGFR and MAPK pathway (Vitagliano, et al. 2011).

The activity profile of this drug made it a good choice as a treatment for patients with unresectable MTC. A phase II clinical trial assessed the efficacy of vandetanib (300 mg once-daily) in patients with advanced hereditary MTC. A total of 30 patients were enrolled; a partial response was achieved in 20% of these patients and durable stable disease for ≥ 24 weeks was reported in 53 % of the patients. Therefore, disease control rate was 73% and serum calcitonin levels decreased 50% or more in 80% of the patients. The most common adverse events were diarrhea (70%), rash (67%), fatigue (63%) and nausea (63%). Others adverse events related to vandetanib included hypertension and QTc prolongation (Wells, et al. 2010). Similar results were described in 19 patients with metastatic hereditary MTC receiving 100 mg/day vandetanib, where the disease control rate was 68% (Robinson, et al. 2010). However, no direct comparison of the efficacy at each dose level - 100 or 300 mg/day - has been conducted.

A recent study evaluated the endocrine effects of vandetanib in patients with progressive thyroid cancer. The main effects observed during treatment were increase of replacement doses of thyroid hormone, calcium and vitamin D analog (Brassard, et al. 2011).

More recently, in a large trial, 331 adults with metastatic MTC (90% with sporadic disease) were randomized to receive either ZD6474 at a dose of 300 mg daily or placebo. A significant

improvement in progression-free survival was observed for patients randomized to receive vandetanib (hazard ratio = 0.46, 95% CI 0.31–0.69). The rate of mortality in two-year follow-up was 15%. A subgroup analysis of the progression-free survival, in sporadic MTC patients, suggested that *RET* M918T mutation–positive patients had a higher response rate to vandetanib compared with *RET* M918T mutation–negative patients (Wells, et al. 2012).

Based on these results, the FDA recommended the approval of vandetanib for the treatment of patients with unresectable or metastatic medullary thyroid cancer - www.fda.gov (Deshpande, et al. 2011). Meanwhile, it is important to emphasize that preclinical studies have evidenced that *RET* activating mutations at codon 804 (V804L, V804M) cause resistance to some TKI, such as vandetanib (Carlomagno, et al. 2004).

Cabozantinib (XL184)

XL184 is a potent inhibitor of MET, VEGFR 2 and RET. A phase I study of XL184 (maximum-tolerated dose 175 md daily) was conducted in 37 patients with MTC. Overall, 68% of patients had a stable disease for 6 months or longer or confirmed partial response (Kurzrock, et al. 2011). Nowadays, a phase III study with XL184 versus placebo in metastatic MTC is ongoing - www.clinicaltrials.gov. At the Annual Meeting of the American Society of Clinical Oncology 2012, the interim analysis of this study showed that the cabozantinib treatment resulted in prolongation of progression free survival when compared wih placebo (11.2 *vs* 4.0 months, respectively).

A recent study compared the effect of four TKI (axitinib, sunitinib, vandetanib, and XL184) on cell proliferation, RET autophosphorylation, and ERK activation in three cell lines: MZ-CRC-1 (M918T *RET* mutation), MTC-TT (C634W *RET* mutation) and TPC-1 (*RET*/PTC-1 rearrangement) cells. The results showed that all four TKI were capable of reducing cell proliferation, yet XL184 was the most efficient inhibitor for MEN2A and vandetanib was the most potent inhibitor for MEN2B (Verbeek, et al. 2011). These data suggest that the use of specific treatments for each mutation could provide additional benefits in the management of metastatic MTC.

Motesanib (AMG 706)

Motesanib is a multi-kinase inhibitor that targets VEGFR 1,2 and 3, PDGFR and stem cell factor receptor (c-Kit). In a previous study, this compound potently inhibited angiogenesis in a variety of *in vivo* models and it was able to induce regressions of large established tumor xenografts

(Polverino, et al. 2006). Recently, the effects of motesanib on wild-type and mutant *Ret* activity in a mouse model of MTC were described. Treatment with motesanib resulted in substantial inhibition of Ret tyrosine phosphorylation and VEGFR2 phosphorylation in TT tumor cell xenografts (Coxon, et al. 2011).

A single-arm study, phase II, investigated the efficacy of motesanib (125 mg once daily) in 91 patients with advanced MTC. Eighty-one percent of patients had stable disease (SD) and 48% had durable SD (≥24 weeks); however, the overall response rate observed was only 2%. The clinical benefit rate was 51% (objective response and durable SD). The most common adverse events were diarrhea (41%), fatigue (41%) hypothyroidism (29%) and hypertension (27%) (Schlumberger, et al. 2009). Another study found that changes from baseline in serum placental growth factor (PIGF) and soluble VEGFR 2 levels, after initiation of therapy with motesanib, predicted the therapeutic response in patients with metastatic medullary thyroid cancer (Bass, et al. 2010).

Sorafenib (BAY-43-9006)

Sorafenib is a multikinase inhibitor with potent activity against RAF and RTKs. Sorafenib inhibits oncogenic RET kinase activity in NIH3T3 cells while induces growth arrest in TT cells (C634R *RET* mutation–positive MTC cell line). Moreover, sorafenib inhibits the growth of cells carrying *RET* V804L or *RET* V804M, both mutants that are resistant to others TKI (Carlomagno, et al. 2006). In cell-based assays, sorafenib exhibits potent inhibition of several RTKs involved in tumor angiogenesis and is able to block autophosphorylation of VEGFR-2, VEGFR-3, PDGFR, Flt-3, and c-Kit (Wilhelm, et al. 2004).

A small observacional study, investigated the efficacy of sorafenib in 5 patients with progressive MTC; after 6 months, 2 patients showed a partial response and 2 patients exhibited stable disease (Frank-Raue, et al. 2011). In a phase II trial, 21 patients with metastatic or locally advanced MTC, hereditary or sporadic form, were enrolled to receive 400mg orally twice daily of sorafenib. The hereditary arm of the study was prematurely closed and it was therefore not possible to conclude on the effect of sorafenib; in the sporadic MTC group, 50% of the patients demonstrated durable stable disease ≥ 15 months and only one partial response (6%). Eleven patients had a decrease in calcitonin and carcinoembryonic antigen (CEA). Sixteen patients required a dose reduction due to adverse events that included rash, diarrhea, hypertension, hand-foot-skin reaction and cytopenias and one death was reported (Lam et al. 2010). More recently, another phase II trial examined a total of 15 patients with metastatic MTC treated with sorafenib. The radiological

response rate was achieved for 25% of patients and similar adverse events were described (Ahmed, et al. 2011).

To investigate the hypothesis that combinations of drugs with different therapeutic targets are synergistically effective and thereby it could be a better option to treat thyroid malignancies, the combination of sorafenib and tipifarnib - a selective oral farnesyltransferase inhibitor - was employed in a phase I trial. Of the 35 patients studied, 13 had MTC and 22 differentiated thyroid cancer. The MTC partial response rate was 38% and the stable disease rate, of at least 6 months, was 31% (Hong, et al. 2011). More recently a synergistic effect of sorafenib and AZD6244(a MEK inhibitor) was demonstrated in the inhibition of human MTC cells, in vitro (Koh, et al. 2012). Despite the limitations in comparing different studies, it seems that combined treatment offers higher rates of partial response than the use of sorafenib only.

Sorafenib is currently approved by the US Food and Drug Administration (FDA) for renal cell and hepatocellular carcinomas.

Sunitinib (SU11248)

Sunitinib is a small molecule that inhibits members of the RTKs family including the VEGFR-1, VEGFR-2, PDGFR, c-Kit and RET (Broutin, et al. 2011; Chow and Eckhardt 2007; Schueneman, et al. 2003).

Recently, two patients with metastatic MTC received sunitinib (50 mg/d, for 28 days, followed by 14 days of no treatment) with a satisfactory response (Cleary, et al. 2010; Kelleher and McDermott 2008). In a phase II study, thirty-five patients with advanced thyroid cancer – seven of them with MTC- received sunitinib at a dose of 37.5 mg daily. The objective response included 1 complete response (3%), 10 partial responses (28%) and 16 patients (46%) with stable disease. The most common toxicities included fatigue, neutropenia, hand-foot syndrome, diarrhea and leucopenia (Carr, et al. 2010).

Currently ongoing, a phase II trial aims to determine the efficacy of sunitinib in patients with locally advanced or metastatic thyroid cancer. The partial results of the 15 patients with MTC show 33.3% partial response and 26.7% stable disease for ≥12 weeks (Ravaud, et al. 2010).

The FDA has approved sunitinib for treatment of advanced renal cell carcinoma and gastrointestinal stromal sarcomas.

Axitinib (AG-013736)

Axitinib is an oral tyrosine kinase inhibitor with a selectivity and potency against VEGFR-1, VEGFR-2 and VEGFR-3 (Inai, et al. 2004). A multicenter, open-label phase II study of the 60 patients with advanced thyroid cancer, of whom 18% had MTC, was conducted using 5 mg daily of axitinib. In MTC patients only, the confirmed partial response rate was 18% and stable disease rate was 27%. Common adverse events included fatigue, stomatitis, proteinuria, diarrhea, hypertension, and nausea (Cohen, et al. 2008).

Imatinib (STI571)

Imatinib is a TKI used to treat chronic myelogenous leukemia (CML) and gastrointestinal stromal tumors. In MTC-derived cell lines expressing mutant RET receptors, imatinib inhibited RET Y1062 phosphorylation and induced cell-cycle arrest and apoptoptic cell death. However, the IC_{50} of imatinib (the concentration that causes 50% growth inhibition) necessary to inhibit RET *in vitro* is higher than other small-molecule kinase inhibitors of RET activity (de Groot, et al. 2006b). An open-label trial evaluated nine patients with unresectable and progressive MTC treated with imatinib (600 mg daily) for 12 months. A complete or partial response was not seen; after 6 months five patients had stable disease and after 12 months only one. The most significant adverse effects were diarrhea, vomiting, abdominal pain and facial edema (Frank-Raue, et al. 2007). Similar results were found in another clinical trial with imatinib in the same doses. Of the 15 patients with disseminated MTC treated for up to 12 months, 4 patients had stable disease over 24 months. Furthermore, imatinib induced considerable toxicity in patients with MTC. The most frequently observed toxicity was hypothyroidism (reverted by increasing doses of thyroid hormone); other adverse events observed were fatigue, nausea and vomiting, trombocytopenia, rash, malaise, and laryngeal mucosal swelling. (de Groot, et al. 2007).

Conclusion

Patients with advanced or metastatic MTC receive only palliative care to relieve disabling symptoms, since the chemotherapy and radiotherapy have unsatisfactory results. Currently, greater knowledge of molecules and intracellular signaling pathways involved in the pathogenesis of MTC has allowed the use of new targeted therapies. Different TKI have been studied in the management of metastatic MTC. The main results demonstrated that TKI are able to induce partial responses or stabilization of tumor growth. However, it is important to remember that TKI also interact with physiological functions causing a number of highly toxic side effects. Moreover, most of the clinical trials were performed on a small number of patients with a brief follow-up period, since tumor growth is very slow in MTC. Sunitinib and sorafenib are now available commercially for the treatment of other cancers and, more recently, FDA recommended the approval of vandetanib for the treatment of patients with unresectable or metastatic MTC. Therefore it is essential to select patients carefully for such therapies. Improved knowledge about the targets of action of these drugs may help choose the best drug for each patient and the best combination to be used to enhance the benefits.

References

Ahmed M, Barbachano Y, Riddell A, Hickey J, Newbold KL, Viros A, Harrington KJ, Marais R & Nutting CM 2011 Analysis of the efficacy and toxicity of sorafenib in thyroid cancer: a phase II study in a UK based population. *Eur J Endocrinol* **165** 315-322.

Airaksinen MS & Saarma M 2002 The GDNF family: signalling, biological functions and therapeutic value. *Nat Rev Neurosci* **3** 383-394.

Arthan D, Hong SK & Park JI 2010 Leukemia inhibitory factor can mediate Ras/Raf/MEK/ERK-induced growth inhibitory signaling in medullary thyroid cancer cells. *Cancer Lett* **297** 31-41.

Asai N, Jijiwa M, Enomoto A, Kawai K, Maeda K, Ichiahara M, Murakumo Y & Takahashi M 2006 RET receptor signaling: dysfunction in thyroid cancer and Hirschsprung's disease. *Pathol Int* **56** 164-172.

Asai N, Murakami H, Iwashita T & Takahashi M 1996 A mutation at tyrosine 1062 in MEN2A-Ret and MEN2B-Ret impairs their transforming activity and association with shc adaptor proteins. *J Biol Chem* **271** 17644-17649.

Auernhammer CJ & Melmed S 2000 Leukemia-inhibitory factor-neuroimmune modulator of endocrine function. *Endocr Rev* **21** 313-345.

Basolo F, Pollina L, Fontanini G, Fiore L, Pacini F & Baldanzi A 1997 Apoptosis and proliferation in thyroid carcinoma: correlation with bcl-2 and p53 protein expression. *Br J Cancer* **75** 537-541.

Bass MB, Sherman SI, Schlumberger MJ, Davis MT, Kivman L, Khoo HM, Notari KH, Peach M, Hei YJ & Patterson SD 2010 Biomarkers as predictors of response to treatment with motesanib in patients with progressive advanced thyroid cancer. *J Clin Endocrinol Metab* **95** 5018-5027.

Baud V & Karin M 2009 Is NF-kappaB a good target for cancer therapy? Hopes and pitfalls. *Nat Rev Drug Discov* **8** 33-40.

Bergholm U, Adami HO, Bergstrom R, Johansson H, Lundell G, Telenius-Berg M & Akerstrom G 1989 Clinical characteristics in sporadic and familial medullary thyroid carcinoma. A nationwide study of 249 patients in Sweden from 1959 through 1981. *Cancer* **63** 1196-1204.

Bjornsti MA & Houghton PJ 2004 The TOR pathway: a target for cancer therapy. *Nat Rev Cancer* **4** 335-348.

Bockhorn M, Frilling A, Kalinin V, Schroder S & Broelsch CE 2000 Absence of H- and K-ras oncogene mutations in sporadic medullary thyroid carcinoma. *Exp Clin Endocrinol Diabetes* **108** 49-53.

Borrego S, Eng C, Sanchez B, Saez ME, Navarro E & Antinolo G 1998 Molecular analysis of the ret and GDNF genes in a family with multiple endocrine neoplasia type 2A and Hirschsprung disease. *J Clin Endocrinol Metab* **83** 3361-3364.

Borrello MG, Smith DP, Pasini B, Bongarzone I, Greco A, Lorenzo MJ, Arighi E, Miranda C, Eng C, Alberti L, et al. 1995 RET activation by germline MEN2A and MEN2B mutations. *Oncogene* **11** 2419-2427.

Bottaro DP, Rubin JS, Faletto DL, Chan AM, Kmiecik TE, Vande Woude GF & Aaronson SA 1991 Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. *Science* **251** 802-804.

Braig M & Schmitt CA 2006 Oncogene-induced senescence: putting the brakes on tumor development. *Cancer Res* **66** 2881-2884.

Brassard M, Neraud B, Trabado S, Salenave S, Brailly-Tabard S, Borget I, Baudin E, Leboulleux S, Chanson P, Schlumberger M, et al. 2011 Endocrine effects of the tyrosine kinase inhibitor vandetanib in patients treated for thyroid cancer. *J Clin Endocrinol Metab* **96** 2741-2749.

Brembeck FH, Rosario M & Birchmeier W 2006 Balancing cell adhesion and Wnt signaling, the key role of beta-catenin. *Curr Opin Genet Dev* **16** 51-59.

Brierley J, Tsang R, Simpson WJ, Gospodarowicz M, Sutcliffe S & Panzarella T 1996 Medullary thyroid cancer: analyses of survival and prognostic factors and the role of radiation therapy in local control. *Thyroid* **6** 305-310.

Broekman F, Giovannetti E & Peters GJ 2011 Tyrosine kinase inhibitors: Multi-targeted or single-targeted? *World J Clin Oncol* **2** 80-93.

Broutin S, Ameur N, Lacroix L, Robert T, Petit B, Oumata N, Talbot M, Caillou B, Schlumberger M, Dupuy C, et al. 2011 Identification of soluble candidate biomarkers of therapeutic response to sunitinib in medullary thyroid carcinoma in preclinical models. *Clin Cancer Res* **17** 2044-2054.

Capp C, Wajner SM, Siqueira DR, Brasil BA, Meurer L & Maia AL 2010 Increased expression of vascular endothelial growth factor and its receptors, VEGFR-1 and VEGFR-2, in medullary thyroid carcinoma. *Thyroid* **20** 863-871.

Carlomagno F, Anaganti S, Guida T, Salvatore G, Troncone G, Wilhelm SM & Santoro M 2006 BAY 43-9006 inhibition of oncogenic RET mutants. *J Natl Cancer Inst* **98** 326-334.

Carlomagno F, Guida T, Anaganti S, Vecchio G, Fusco A, Ryan AJ, Billaud M & Santoro M 2004 Disease associated mutations at valine 804 in the RET receptor tyrosine kinase confer resistance to selective kinase inhibitors. *Oncogene* **23** 6056-6063.

Carlomagno F, Vitagliano D, Guida T, Ciardiello F, Tortora G, Vecchio G, Ryan AJ, Fontanini G, Fusco A & Santoro M 2002 ZD6474, an orally available inhibitor of KDR tyrosine kinase activity, efficiently blocks oncogenic RET kinases. *Cancer Res* **62** 7284-7290.

Carr LL, Mankoff DA, Goulart BH, Eaton KD, Capell PT, Kell EM, Bauman JE & Martins RG 2010 Phase II study of daily sunitinib in FDG-PET-positive, iodine-refractory differentiated thyroid cancer and metastatic medullary carcinoma of the thyroid with functional imaging correlation. *Clin Cancer Res* **16** 5260-5268.

Cassinelli G, Favini E, Degl'Innocenti D, Salvi A, De Petro G, Pierotti MA, Zunino F, Borrello MG & Lanzi C 2009 RET/PTC1-driven neoplastic transformation and proinvasive phenotype of human thyrocytes involve Met induction and beta-catenin nuclear translocation. *Neoplasia* **11** 10-21.

Castellone MD, De Falco V, Rao DM, Bellelli R, Muthu M, Basolo F, Fusco A, Gutkind JS & Santoro M 2009 The beta-catenin axis integrates multiple signals downstream from RET/papillary thyroid carcinoma leading to cell proliferation. *Cancer Res* **69** 1867-1876.

Cecchi F, Rabe DC & Bottaro DP 2010 Targeting the HGF/Met signalling pathway in cancer. *Eur J Cancer* **46** 1260-1270.

Ceolin L, Siqueira DR, Romitti M, Ferreira CV & Maia AL 2012 Molecular Basis of Medullary Thyroid Carcinoma: The Role of RET Polymorphisms. *Int J Mol Sci* **13** 221-239.

Cerrato A, De Falco V & Santoro M 2009 Molecular genetics of medullary thyroid carcinoma: the quest for novel therapeutic targets. *J Mol Endocrinol* **43** 143-155.

Chow LQ & Eckhardt SG 2007 Sunitinib: from rational design to clinical efficacy. *J Clin Oncol* **25** 884-896.

Cleary JM, Sadow PM, Randolph GW, Palmer EL, Lynch TP, Nikiforov YE & Wirth LJ 2010 Neoadjuvant treatment of unresectable medullary thyroid cancer with sunitinib. *J Clin Oncol* **28** e390-392.

Cohen EE, Rosen LS, Vokes EE, Kies MS, Forastiere AA, Worden FP, Kane MA, Sherman E, Kim S, Bycott P, et al. 2008 Axitinib is an active treatment for all histologic subtypes of advanced thyroid cancer: results from a phase II study. *J Clin Oncol* **26** 4708-4713.

Coxon A, Bready J, Kaufman S, Estrada J, Osgood T, Canon J, Wang L, Radinsky R, Kendall R, Hughes P, et al. 2011 Antitumor Activity of Motesanib in a Medullary Thyroid Cancer Model. *J Endocrinol Invest*.

Croyle M, Akeno N, Knauf JA, Fabbro D, Chen X, Baumgartner JE, Lane HA & Fagin JA 2008 RET/PTC-induced cell growth is mediated in part by epidermal growth factor receptor (EGFR) activation: evidence for molecular and functional interactions between RET and EGFR. *Cancer Res* **68** 4183-4191.

de Groot JW, Links TP, Plukker JT, Lips CJ & Hofstra RM 2006a RET as a diagnostic and therapeutic target in sporadic and hereditary endocrine tumors. *Endocr Rev* **27** 535-560.

de Groot JW, Plaza Menacho I, Schepers H, Drenth-Diephuis LJ, Osinga J, Plukker JT, Links TP, Eggen BJ & Hofstra RM 2006b Cellular effects of imatinib on medullary thyroid cancer cells harboring multiple endocrine neoplasia Type 2A and 2B associated RET mutations. *Surgery* **139** 806-814.

de Groot JW, Zonnenberg BA, van Ufford-Mannesse PQ, de Vries MM, Links TP, Lips CJ & Voest EE 2007 A phase II trial of imatinib therapy for metastatic medullary thyroid carcinoma. *J Clin Endocrinol Metab* **92** 3466-3469.

de la Torre NG, Buley I, Wass JA & Turner HE 2006 Angiogenesis and lymphangiogenesis in thyroid proliferative lesions: relationship to type and tumour behaviour. *Endocr Relat Cancer* **13** 931-944.

Deshpande H, Roman S, Thumar J & Sosa JA 2011 Vandetanib (ZD6474) in the Treatment of Medullary Thyroid Cancer. *Clin Med Insights Oncol* **5** 213-221.

Donis-Keller H, Dou S, Chi D, Carlson KM, Toshima K, Lairmore TC, Howe JR, Moley JF, Goodfellow P & Wells SA, Jr. 1993 Mutations in the RET proto-oncogene are associated with MEN 2A and FMTC. *Hum Mol Genet* **2** 851-856.

Elisei R, Cosci B, Romei C, Bottici V, Renzini G, Molinaro E, Agate L, Vivaldi A, Faviana P, Basolo F, et al. 2008 Prognostic significance of somatic RET oncogene mutations in sporadic medullary thyroid cancer: a 10-year follow-up study. *J Clin Endocrinol Metab* **93** 682-687.

Encinas M, Rozen EJ, Dolcet X, Jain S, Comella JX, Milbrandt J & Johnson EM, Jr. 2008 Analysis of Ret knockin mice reveals a critical role for IKKs, but not PI 3-K, in neurotrophic factor-induced survival of sympathetic neurons. *Cell Death Differ* **15** 1510-1521.

Frank-Raue K, Fabel M, Delorme S, Haberkorn U & Raue F 2007 Efficacy of imatinib mesylate in advanced medullary thyroid carcinoma. *Eur J Endocrinol* **157** 215-220.

Frank-Raue K, Ganten M, Kreissl MC & Raue F 2011 Rapid response to sorafenib in metastatic medullary thyroid carcinoma. *Exp Clin Endocrinol Diabetes* **119** 151-155.

Fresno Vara JA, Casado E, de Castro J, Cejas P, Belda-Iniesta C & Gonzalez-Baron M 2004 PI3K/Akt signalling pathway and cancer. *Cancer Treat Rev* **30** 193-204.

Gallel P, Pallares J, Dolcet X, Llobet D, Eritja N, Santacana M, Yeramian A, Palomar-Asenjo V, Lagarda H, Mauricio D, et al. 2008 Nuclear factor-kappaB activation is associated with somatic and germ line RET mutations in medullary thyroid carcinoma. *Hum Pathol* **39** 994-1001.

Giraudet AL, Vanel D, Leboulleux S, Auperin A, Dromain C, Chami L, Ny Tovo N, Lumbroso J, Lassau N, Bonniaud G, et al. 2007 Imaging medullary thyroid carcinoma with persistent elevated calcitonin levels. *J Clin Endocrinol Metab* **92** 4185-4190.

Girelli ME, Nacamulli D, Pelizzo MR, De Vido D, Mian C, Piccolo M & Busnardo B 1998 Medullary thyroid carcinoma: clinical features and long-term follow-up of seventy-eight patients treated between 1969 and 1986. *Thyroid* **8** 517-523.

Gorla L, Mondellini P, Cuccuru G, Micciche F, Cassinelli G, Cremona M, Pierotti MA, Lanzi C & Bongarzone I 2009 Proteomics study of medullary thyroid carcinomas expressing RET germ-line mutations: identification of new signaling elements. *Mol Carcinog* **48** 220-231.

Goutas N, Vlachodimitropoulos D, Bouka M, Lazaris AC, Nasioulas G & Gazouli M 2008 BRAF and K-RAS mutation in a Greek papillary and medullary thyroid carcinoma cohort. *Anticancer Res* **28** 305-308.

Gujral TS, van Veelen W, Richardson DS, Myers SM, Meens JA, Acton DS, Dunach M, Elliott BE, Hoppener JW & Mulligan LM 2008 A novel RET kinase-beta-catenin signaling pathway contributes to tumorigenesis in thyroid carcinoma. *Cancer Res* **68** 1338-1346.

Hahn WC & Weinberg RA 2002 Modelling the molecular circuitry of cancer. *Nat Rev Cancer* **2** 331-341.

Halilovic E & Solit DB 2008 Therapeutic strategies for inhibiting oncogenic BRAF signaling. *Curr Opin Pharmacol* **8** 419-426.

Harris TJ & Peifer M 2005 Decisions, decisions: beta-catenin chooses between adhesion and transcription. *Trends Cell Biol* **15** 234-237.

Harvey M, Vogel H, Lee EY, Bradley A & Donehower LA 1995 Mice deficient in both p53 and Rb develop tumors primarily of endocrine origin. *Cancer Res* **55** 1146-1151.

Herfarth KK, Wick MR, Marshall HN, Gartner E, Lum S & Moley JF 1997 Absence of TP53 alterations in pheochromocytomas and medullary thyroid carcinomas. *Genes Chromosomes Cancer* **20** 24-29.

Hong DS, Cabanillas ME, Wheler J, Naing A, Tsimberidou AM, Ye L, Waguespack SG, Hernandez M, El Naggar AK, Bidyasar S, et al. 2011 Inhibition of the Ras/Raf/MEK/ERK and RET kinase pathways with the combination of the multikinase inhibitor sorafenib and the farnesyltransferase inhibitor tipifarnib in medullary and differentiated thyroid malignancies. *J Clin Endocrinol Metab* **96** 997-1005.

Hong SK, Yoon S, Moelling C, Arthan D & Park JI 2009 Noncatalytic function of ERK1/2 can promote Raf/MEK/ERK-mediated growth arrest signaling. *J Biol Chem* **284** 33006-33018.

Ichihara M, Murakumo Y & Takahashi M 2004 RET and neuroendocrine tumors. *Cancer Lett* **204** 197-211.

Inai T, Mancuso M, Hashizume H, Baffert F, Haskell A, Baluk P, Hu-Lowe DD, Shalinsky DR, Thurston G, Yancopoulos GD, et al. 2004 Inhibition of vascular endothelial growth factor (VEGF) signaling in cancer causes loss of endothelial fenestrations, regression of tumor vessels, and appearance of basement membrane ghosts. *Am J Pathol* **165** 35-52.

Ishizaka Y, Itoh F, Tahira T, Ikeda I, Sugimura T, Tucker J, Fertitta A, Carrano AV & Nagao M 1989 Human ret proto-oncogene mapped to chromosome 10q11.2. *Oncogene* **4** 1519-1521.

Iwashita T, Asai N, Murakami H, Matsuyama M & Takahashi M 1996 Identification of tyrosine residues that are essential for transforming activity of the ret proto-oncogene with MEN2A or MEN2B mutation. *Oncogene* **12** 481-487.

Joshi PP, Kulkarni MV, Yu BK, Smith KR, Norton DL, van Veelen W, Hoppener JW & Franklin DS 2007 Simultaneous downregulation of CDK inhibitors p18(Ink4c) and p27(Kip1) is required for MEN2A-RET-mediated mitogenesis. *Oncogene* **26** 554-570.

Kauffmann-Zeh A, Rodriguez-Viciana P, Ulrich E, Gilbert C, Coffer P, Downward J & Evan G 1997 Suppression of c-Myc-induced apoptosis by Ras signalling through PI(3)K and PKB. *Nature* **385** 544-548.

Kebebew E, Ituarte PH, Siperstein AE, Duh QY & Clark OH 2000 Medullary thyroid carcinoma: clinical characteristics, treatment, prognostic factors, and a comparison of staging systems. *Cancer* **88** 1139-1148.

Kelleher FC & McDermott R 2008 Response to sunitinib in medullary thyroid cancer. *Ann Intern Med* **148** 567.

Knauf JA & Fagin JA 2009 Role of MAPK pathway oncoproteins in thyroid cancer pathogenesis and as drug targets. *Curr Opin Cell Biol* **21** 296-303.

Kodama Y, Asai N, Kawai K, Jijiwa M, Murakumo Y, Ichihara M & Takahashi M 2005 The RET proto-oncogene: a molecular therapeutic target in thyroid cancer. *Cancer Sci* **96** 143-148.

Koh YW, Shah MH, Agarwal K, McCarty SK, Koo BS, Brendel VJ, Wang C, Porter K, Jarjoura D, Saji M, et al. 2012 Sorafenib and Mek inhibition is synergistic in medullary thyroid carcinoma in vitro. *Endocr Relat Cancer* **19** 29-38.

Kouvaraki MA, Shapiro SE, Perrier ND, Cote GJ, Gagel RF, Hoff AO, Sherman SI, Lee JE & Evans DB 2005 RET proto-oncogene: a review and update of genotype-phenotype correlations in hereditary medullary thyroid cancer and associated endocrine tumors. *Thyroid* **15** 531-544.

Kunnimalaiyaan M, Ndiaye M & Chen H 2006 Apoptosis-mediated medullary thyroid cancer growth suppression by the PI3K inhibitor LY294002. *Surgery* **140** 1009-1014; discussion 1014-1005.

Kurzrock R, Sherman SI, Ball DW, Forastiere AA, Cohen RB, Mehra R, Pfister DG, Cohen EE, Janisch L, Nauling F, et al. 2011 Activity of XL184 (Cabozantinib), an oral tyrosine kinase inhibitor, in patients with medullary thyroid cancer. *J Clin Oncol* **29** 2660-2666.

Lam ET, Ringel MD, Kloos RT, Prior TW, Knopp MV, Liang J, Sammet S, Hall NC, Wakely PE, Jr., Vasko VV, et al. 2010 Phase II clinical trial of sorafenib in metastatic medullary thyroid cancer. *J Clin Oncol* **28** 2323-2330.

Licitra L, Locati LD, Greco A, Granata R & Bossi P 2010 Multikinase inhibitors in thyroid cancer. *Eur J Cancer* **46** 1012-1018.

Ludwig L, Kessler H, Wagner M, Hoang-Vu C, Dralle H, Adler G, Bohm BO & Schmid RM 2001 Nuclear factor-kappaB is constitutively active in C-cell carcinoma and required for RET-induced transformation. *Cancer Res* **61** 4526-4535.

Machens A & Dralle H 2010 Parenchymal versus nonparenchymal target lesion response in clinical trials for metastatic medullary thyroid cancer. *J Clin Oncol* **28** e534: author reply e535-536.

Marsh DJ, Learoyd DL, Andrew SD, Krishnan L, Pojer R, Richardson AL, Delbridge L, Eng C & Robinson BG 1996 Somatic mutations in the RET proto-oncogene in sporadic medullary thyroid carcinoma. *Clin Endocrinol (Oxf)* **44** 249-257.

Marsh DJ, Zheng Z, Arnold A, Andrew SD, Learoyd D, Frilling A, Komminoth P, Neumann HP, Ponder BA, Rollins BJ, et al. 1997 Mutation analysis of glial cell line-derived neurotrophic factor, a ligand for an RET/coreceptor complex, in multiple endocrine neoplasia type 2 and sporadic neuroendocrine tumors. *J Clin Endocrinol Metab* **82** 3025-3028.

Melillo RM, Barone MV, Lupoli G, Cirafici AM, Carlomagno F, Visconti R, Matoskova B, Di Fiore PP, Vecchio G, Fusco A, et al. 1999 Ret-mediated mitogenesis requires Src kinase activity. *Cancer Res* **59** 1120-1126.

Mitsiades CS, Kotoula V, Poulaki V, Sozopoulos E, Negri J, Charalambous E, Fanourakis G, Voutsinas G, Tseleni-Balafouta S & Mitsiades N 2006a Epidermal growth factor receptor as a therapeutic target in human thyroid carcinoma: mutational and functional analysis. *J Clin Endocrinol Metab* **91** 3662-3666.

Mitsiades CS, McMillin D, Kotoula V, Poulaki V, McMullan C, Negri J, Fanourakis G, Tseleni-Balafouta S, Ain KB & Mitsiades N 2006b Antitumor effects of the proteasome inhibitor bortezomib in medullary and anaplastic thyroid carcinoma cells in vitro. *J Clin Endocrinol Metab* **91** 4013-4021.

Moley JF, Brother MB, Wells SA, Spengler BA, Biedler JL & Brodeur GM 1991 Low frequency of ras gene mutations in neuroblastomas, pheochromocytomas, and medullary thyroid cancers. *Cancer Res* **51** 1596-1599.

Moley JF & DeBenedetti MK 1999 Patterns of nodal metastases in palpable medullary thyroid carcinoma: recommendations for extent of node dissection. *Ann Surg* **229** 880-887; discussion 887-888.

Morin PJ 1999 beta-catenin signaling and cancer. *Bioessays* **21** 1021-1030.

Moura MM, Cavaco BM, Pinto AE, Domingues R, Santos JR, Cid MO, Bugalho MJ & Leite V 2009 Correlation of RET somatic mutations with clinicopathological features in sporadic medullary thyroid carcinomas. *Br J Cancer* **100** 1777-1783.

Moura MM, Cavaco BM, Pinto AE & Leite V 2011 High prevalence of RAS mutations in RET-negative sporadic medullary thyroid carcinomas. *J Clin Endocrinol Metab* **96** E863-868.

Mulligan LM, Kwok JB, Healey CS, Elsdon MJ, Eng C, Gardner E, Love DR, Mole SE, Moore JK, Papi L, et al. 1993 Germ-line mutations of the RET proto-oncogene in multiple endocrine neoplasia type 2A. *Nature* **363** 458-460.

Namba H, Saenko V & Yamashita S 2007 Nuclear factor-kB in thyroid carcinogenesis and progression: a novel therapeutic target for advanced thyroid cancer. *Arq Bras Endocrinol Metabol* **51** 843-851.

Ning L, Kunnimalaiyaan M & Chen H 2008 Regulation of cell-cell contact molecules and the metastatic phenotype of medullary thyroid carcinoma by the Raf-1/MEK/ERK pathway. *Surgery* **144** 920-924; discussion 924-925.

Nocera M, Baudin E, Pellegriti G, Cailleux AF, Mechelany-Corone C & Schlumberger M 2000 Treatment of advanced medullary thyroid cancer with an alternating combination of doxorubicin-streptozocin and 5 FU-dacarbazine. Groupe d'Etude des Tumeurs a Calcitonine (GETC). *Br J Cancer* **83** 715-718.

Normanno N, De Luca A, Bianco C, Strizzi L, Mancino M, Maiello MR, Carotenuto A, De Feo G, Caponigro F & Salomon DS 2006 Epidermal growth factor receptor (EGFR) signaling in cancer. *Gene* **366** 2-16.

Pacifico F & Leonardi A 2010 Role of NF-kappaB in thyroid cancer. *Mol Cell Endocrinol* **321** 29-35.

Palona I, Namba H, Mitsutake N, Starenki D, Podtcheko A, Sedliarou I, Ohtsuru A, Saenko V, Nagayama Y, Umezawa K, et al. 2006 BRAFV600E promotes invasiveness of thyroid cancer cells through nuclear factor kappaB activation. *Endocrinology* **147** 5699-5707.

Park JI, Strock CJ, Ball DW & Nelkin BD 2003 The Ras/Raf/MEK/extracellular signal-regulated kinase pathway induces autocrine-paracrine growth inhibition via the leukemia inhibitory factor/JAK/STAT pathway. *Mol Cell Biol* **23** 543-554.

Pavelic K, Dedivitis RA, Kapitanovic S, Cacev T, Guirado CR, Danic D, Radosevic S, Brkic K, Pegan B, Krizanac S, et al. 2006 Molecular genetic alterations of FHIT and p53 genes in benign and malignant thyroid gland lesions. *Mutat Res* **599** 45-57.

Pelizzo MR, Boschin IM, Bernante P, Toniato A, Piotto A, Pagetta C, Nibale O, Rampin L, Muzzio PC & Rubello D 2007 Natural history, diagnosis, treatment and outcome of medullary thyroid cancer: 37 years experience on 157 patients. *Eur J Surg Oncol* **33** 493-497.

Plaza Menacho I, Koster R, van der Sloot AM, Quax WJ, Osinga J, van der Sluis T, Hollema H, Burzynski GM, Gimm O, Buys CH, et al. 2005 RET-familial medullary thyroid carcinoma mutants Y791F and S891A activate a Src/JAK/STAT3 pathway, independent of glial cell line-derived neurotrophic factor. *Cancer Res* **65** 1729-1737.

Plotnikov A, Zehorai E, Procaccia S & Seger R 2011 The MAPK cascades: signaling components, nuclear roles and mechanisms of nuclear translocation. *Biochim Biophys Acta* **1813** 1619-1633.

Polverino A, Coxon A, Starnes C, Diaz Z, DeMelfi T, Wang L, Bready J, Estrada J, Cattley R, Kaufman S, et al. 2006 AMG 706, an oral, multikinase inhibitor that selectively targets vascular endothelial growth factor, platelet-derived growth factor, and kit receptors, potently inhibits angiogenesis and induces regression in tumor xenografts. *Cancer Res* **66** 8715-8721.

Punales MK, Graf H, Gross JL & Maia AL 2003 RET codon 634 mutations in multiple endocrine neoplasia type 2: variable clinical features and clinical outcome. *J Clin Endocrinol Metab* **88** 2644-2649.

Ravaud A, de la Fouchardiere C, Asselineau J, Delord JP, Do Cao C, Niccoli P, Rodien P, Klein M & Catargi B 2010 Efficacy of sunitinib in advanced medullary thyroid carcinoma: intermediate results of phase II THYSU. *Oncologist* **15** 212-213; author reply 214.

Robinson BG, Paz-Ares L, Krebs A, Vasselli J & Haddad R 2010 Vandetanib (100 mg) in patients with locally advanced or metastatic hereditary medullary thyroid cancer. *J Clin Endocrinol Metab* **95** 2664-2671.

Roman S, Lin R & Sosa JA 2006 Prognosis of medullary thyroid carcinoma: demographic, clinical, and pathologic predictors of survival in 1252 cases. *Cancer* **107** 2134-2142.

Santoro M, Carlomagno F, Romano A, Bottaro DP, Dathan NA, Grieco M, Fusco A, Vecchio G, Matoskova B, Kraus MH, et al. 1995 Activation of RET as a dominant transforming gene by germline mutations of MEN2A and MEN2B. *Science* **267** 381-383.

Schilling T, Burck J, Sinn HP, Clemens A, Otto HF, Hoppner W, Herfarth C, Ziegler R, Schwab M & Raue F 2001 Prognostic value of codon 918 (ATG-->ACG) RET proto-oncogene mutations in sporadic medullary thyroid carcinoma. *Int J Cancer* **95** 62-66.

Schindler C & Darnell JE, Jr. 1995 Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. *Annu Rev Biochem* **64** 621-651.

Schlessinger J 2000 Cell signaling by receptor tyrosine kinases. *Cell* **103** 211-225.

Schlumberger MJ, Elisei R, Bastholt L, Wirth LJ, Martins RG, Locati LD, Jarzab B, Pacini F, Daumerie C, Droz JP, et al. 2009 Phase II study of safety and efficacy of motesanib in patients with progressive or symptomatic, advanced or metastatic medullary thyroid cancer. *J Clin Oncol* **27** 3794-3801.

Schubbert S, Shannon K & Bollag G 2007 Hyperactive Ras in developmental disorders and cancer. *Nat Rev Cancer* **7** 295-308.

Schueneman AJ, Himmelfarb E, Geng L, Tan J, Donnelly E, Mendel D, McMahon G & Hallahan DE 2003 SU11248 maintenance therapy prevents tumor regrowth after fractionated irradiation of murine tumor models. *Cancer Res* **63** 4009-4016.

Schuringa JJ, Wojtachnio K, Hagens W, Vellenga E, Buys CH, Hofstra R & Kruijer W 2001 MEN2A-RET-induced cellular transformation by activation of STAT3. *Oncogene* **20** 5350-5358.

Scollo C, Baudin E, Travagli JP, Caillou B, Bellon N, Leboulleux S & Schlumberger M 2003 Rationale for central and bilateral lymph node dissection in sporadic and hereditary medullary thyroid cancer. *J Clin Endocrinol Metab* **88** 2070-2075.

Segouffin-Cariou C & Billaud M 2000 Transforming ability of MEN2A-RET requires activation of the phosphatidylinositol 3-kinase/AKT signaling pathway. *J Biol Chem* **275** 3568-3576.

Sheikh HA, Tometsko M, Niehouse L, Aldeeb D, Swalsky P, Finkelstein S, Barnes EL & Hunt JL 2004 Molecular genotyping of medullary thyroid carcinoma can predict tumor recurrence. *Am J Surg Pathol* **28** 101-106.

Sherr CJ & Roberts JM 1999 CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev* **13** 1501-1512.

Shibuya M & Claesson-Welsh L 2006 Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis. *Exp Cell Res* **312** 549-560.

Siqueira DR, Romitti M, da Rocha AP, Ceolin L, Meotti C, Estivalet A, Punales MK & Maia AL 2010 The RET polymorphic allele S836S is associated with early metastatic disease in patients with hereditary or sporadic medullary thyroid carcinoma. *Endocr Relat Cancer* **17** 953-963.

Stahl N, Farruggella TJ, Boulton TG, Zhong Z, Darnell JE, Jr. & Yancopoulos GD 1995 Choice of STATs and other substrates specified by modular tyrosine-based motifs in cytokine receptors. *Science* **267** 1349-1353.

Starenki D, Namba H, Saenko V, Ohtsuru A & Yamashita S 2004 Inhibition of nuclear factor-kappaB cascade potentiates the effect of a combination treatment of anaplastic thyroid cancer cells. *J Clin Endocrinol Metab* **89** 410-418.

Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC, et al. 2000 New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* **92** 205-216.

Trovato M, Villari D, Bartolone L, Spinella S, Simone A, Violi MA, Trimarchi F, Batolo D & Benvenga S 1998 Expression of the hepatocyte growth factor and c-met in normal thyroid, non-neoplastic, and neoplastic nodules. *Thyroid* **8** 125-131.

Trusolino L, Bertotti A & Comoglio PM 2010 MET signalling: principles and functions in development, organ regeneration and cancer. *Nat Rev Mol Cell Biol* **11** 834-848.

van der Laan BF, Freeman JL & Asa SL 1995 Expression of growth factors and growth factor receptors in normal and tumorous human thyroid tissues. *Thyroid* **5** 67-73.

van Veelen W, Klompmaker R, Gloerich M, van Gasteren CJ, Kalkhoven E, Berger R, Lips CJ, Medema RH, Hoppener JW & Acton DS 2009 P18 is a tumor suppressor gene involved in human medullary thyroid carcinoma and pheochromocytoma development. *Int J Cancer* **124** 339-345.

van Veelen W, van Gasteren CJ, Acton DS, Franklin DS, Berger R, Lips CJ & Hoppener JW 2008 Synergistic effect of oncogenic RET and loss of p18 on medullary thyroid carcinoma development. *Cancer Res* **68** 1329-1337.

Verbeek HH, Alves MM, de Groot JW, Osinga J, Plukker JT, Links TP & Hofstra RM 2011 The effects of four different tyrosine kinase inhibitors on medullary and papillary thyroid cancer cells. *J Clin Endocrinol Metab* **96** E991-995.

Vieira JM, Santos SC, Espadinha C, Correia I, Vag T, Casalou C, Cavaco BM, Catarino AL, Dias S & Leite V 2005 Expression of vascular endothelial growth factor (VEGF) and its receptors in thyroid carcinomas of follicular origin: a potential autocrine loop. *Eur J Endocrinol* **153** 701-709.

Visconti R, Cerutti J, Battista S, Fedele M, Trapasso F, Zeki K, Miano MP, de Nigris F, Casalino L, Curcio F, et al. 1997 Expression of the neoplastic phenotype by human thyroid carcinoma cell lines requires NFkappaB p65 protein expression. *Oncogene* **15** 1987-1994.

Vitagliano D, De Falco V, Tamburrino A, Coluzzi S, Troncone G, Chiappetta G, Ciardiello F, Tortora G, Fagin JA, Ryan AJ, et al. 2011 The tyrosine kinase inhibitor ZD6474 blocks proliferation of RET mutant medullary thyroid carcinoma cells. *Endocr Relat Cancer* **18** 1-11.

Wang DG, Liu WH, Johnston CF, Sloan JM & Buchanan KD 1998 Bcl-2 and c-Myc, but not bax and p53, are expressed during human medullary thyroid tumorigenesis. *Am J Pathol* **152** 1407-1413.

Wang W, Johansson HE, Bergholm UI, Westermark KM & Grimelius LE 1997 Expression of c-Myc, TGF-alpha and EGF-receptor in sporadic medullary thyroid carcinoma. *Acta Oncol* **36** 407-411.

Wasenius VM, Hemmer S, Karjalainen-Lindsberg ML, Nupponen NN, Franssila K & Joensuu H 2005 MET receptor tyrosine kinase sequence alterations in differentiated thyroid carcinoma. *Am J Surg Pathol* **29** 544-549.

Wedge SR, Ogilvie DJ, Dukes M, Kendrew J, Chester R, Jackson JA, Boffey SJ, Valentine PJ, Curwen JO, Musgrove HL, et al. 2002 ZD6474 inhibits vascular endothelial growth factor signaling, angiogenesis, and tumor growth following oral administration. *Cancer Res* **62** 4645-4655.

Wells SA, Jr., Gosnell JE, Gagel RF, Moley J, Pfister D, Sosa JA, Skinner M, Krebs A, Vasselli J & Schlumberger M 2010 Vandetanib for the treatment of patients with locally advanced or metastatic hereditary medullary thyroid cancer. *J Clin Oncol* **28** 767-772.

Wells SA, Jr., Robinson BG, Gagel RF, Dralle H, Fagin JA, Santoro M, Baudin E, Elisei R, Jarzab B, Vasselli JR, et al. 2012 Vandetanib in patients with locally advanced or metastatic medullary thyroid cancer: a randomized, double-blind phase III trial. *J Clin Oncol* **30** 134-141.

Wilhelm SM, Carter C, Tang L, Wilkie D, McNabola A, Rong H, Chen C, Zhang X, Vincent P, McHugh M, et al. 2004 BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Res* **64** 7099-7109.

Wu G, Mambo E, Guo Z, Hu S, Huang X, Gollin SM, Trink B, Ladenson PW, Sidransky D & Xing M 2005 Uncommon mutation, but common amplifications, of the PIK3CA gene in thyroid tumors. *J Clin Endocrinol Metab* **90** 4688-4693.

Yuan TL & Cantley LC 2008 PI3K pathway alterations in cancer: variations on a theme. *Oncogene* **27** 5497-5510.

Table 1. Summary of clinical trials with tyrosine kinase inhibitors in medullary thyroid carcinoma

Drug	Targets	No of patients	Drug dose	Partial response (%)	Stable disease (%)	Time of stable disease	Reference
Vandetanib (ZD6474)	VEGFR-1, VEGFR-2, VEGFR-3, RET, EGFR	30	300 mg qd	**20**	**53**	6 months	Wells 2010
		19	100 mg qd	**16**	**53**	6 months	Robinson 2010
		331	300 mg qd	**5.4** [b]	**2.6** [c]	6 months	Wells 2012
Carbozantinib (XL 184)	VEGFR-2, RET, MET	37	175 mg qd	**29**	**41**	6 months	Kurzrock 2011
Montesanib (AMG 706)	VEGFR-1, VEGFR-2, VEGFR-3, C-KIT, RET, PDGFR	91	125 mg qd	**2**	**48**	6 months	Schlumberger 2009
Sorafenib (BAY 43-9006)	VEGFR-2, VEGFR-3, c-Kit, RET	5	400 mg bid	**40**	**40**	6 months	Frank-Raue 2011
		16	400 mg bid	**6**	**50**	15 months	Lam 2010
		15	400 mg bid	**25**	-	12 months	Ahmed 2011
Sunitib (SU 11248)	VEGFR-1, VEGFR-2, VEGFR-3, RET, c-Kit	7	37.5 mg qd	**28**[a]	**46**[a]	15.5 months	Carr 2010
		15	50mg qd, 4 weeks every 6 weeks	**33.3**	**26.7**	6 months	Ravaud 2010
Axitinib (AG-013736)	VEGFR-1, VEGFR-2, VEGFR-3, c-Kit	11	5 mg bid	**18**	**27**	16 weeks	Cohen 2008
Imanitib (STI571)	RET, c-Kit, PDGFR	9	600 mg qd	**0**	**55**	6 months	Frank-Raue 2007
		15	600 mg qd	**0**	**27**	24 months	de Groot 2007

Abbreviations: qd, every day; bid, twice a day. [a] Results for the total number of patients with advanced thyroid cancer, not only MTC patients. [b] Odds ratio of objective response rate (placebo *vs* vandetanib). [c] Odds ratio of disease control rate (placebo *vs* vandetanib).

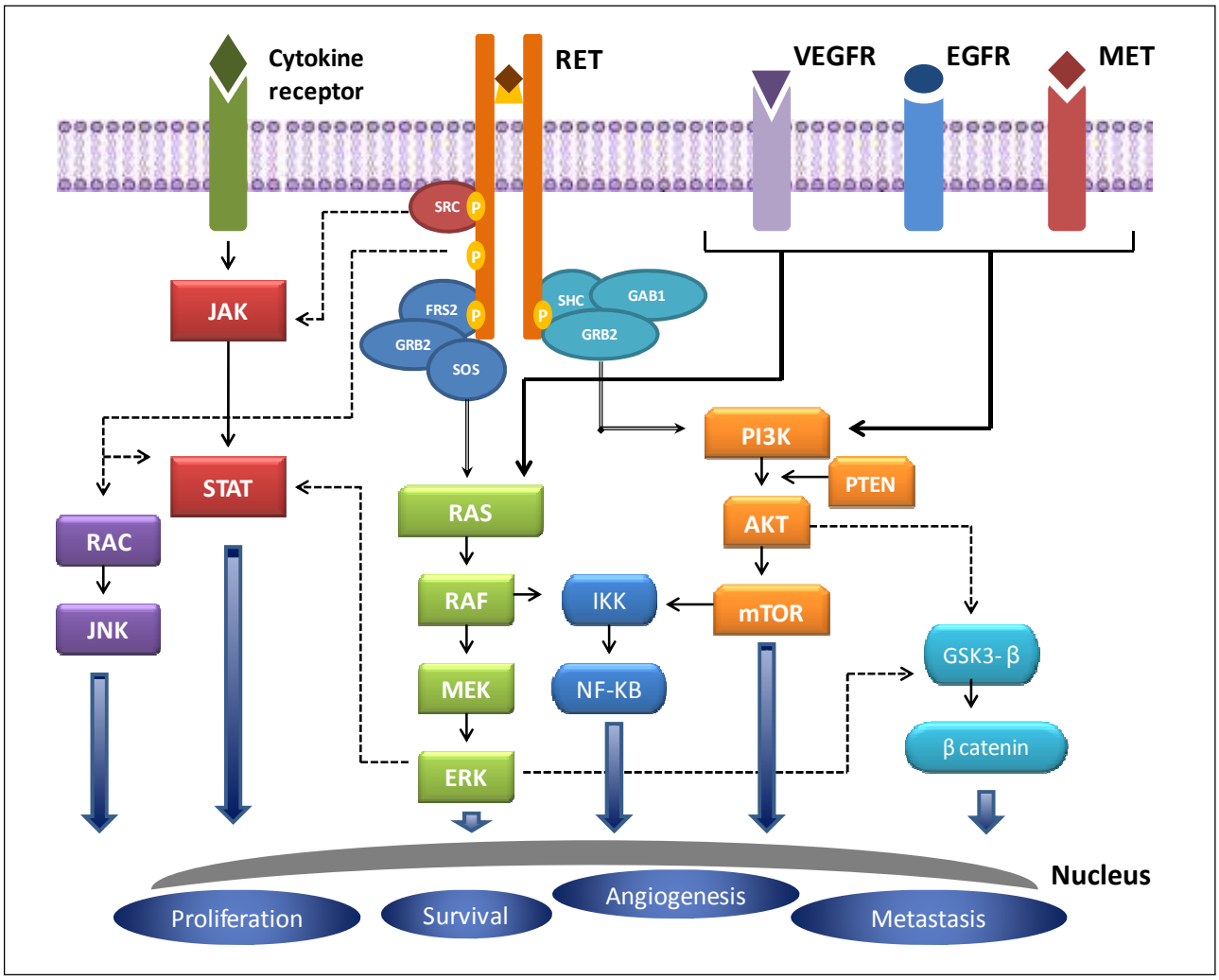

Figure 1. Schematic representation of the intracellular signaling pathways activated by tyrosine kinase receptors in MTC cell. Once activated, these pathways promote cell proliferation, survival and angiogenesis.