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Avaliação dos potenciais mecanismos moleculares associados à variante genética S836S do proto-oncogene *RET* na patogênese do Carcinoma Medular de Tireoide

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- **Artigo de revisão:** Molecular Basis of Medullary Thyroid Carcinoma: The Role of *RET* Polymorphisms; publicado no *Internationa Journal of Molecular Science*. 2012, 13, 221-239; doi:10.3390/ijms13010221

- **Artigo original:** Effect of 3′UTR *RET* Variants on the Secondary Structure of *RET* mRNA in Medullary Thyroid Carcinoma.

Além dos artigos já citados, ao longo do período do doutorado foram desenvolvidos os seguintes manuscritos relacionados ao tema oncogênese tireoidiana:

- Role of VEGF-A and Its Receptors in Sporadic and MEN2-Associated Pheochromocytoma. Ferreira C, Siqueira DR, Romitti M, **Ceolin L**, Brasil B, Meurer L, Capp C, Maia AL. International Journal of Molecular Sciences, v. 15, p. 5323-5336, 2014.

- Role of *RET* genetic variants in MEN 2-associated pheochromocytoma. Siqueira DR, **Ceolin L**, Ferreira CV, Romitti M, Maia SC, Zanini Maciel LM, Maia AL. European Journal of Endocrinology, v. 1, p. 1-10, 2014.

- Advanced medullary thyroid cancer: pathophysiology and management. Ferreira CV, Siqueira DR, **Ceolin L**, Maia AL. Cancer Management and Research, v.5, p. 57–66, 2013.

- Signaling Pathways in Follicular Cell-Derived Thyroid Carcinomas (review); Romitti M, **Ceolin L**, Siqueira DR, Ferreira CV, Wajner SM and Maia AL. International Journal of Oncology; 42(1):19-28, jan 2013.

- Is there a role for inherited TRβ mutation in human carcinogenesis? Weinert LS; **Ceolin L**, Romitti M, Camargo EG, Maia AL. Arquivos Brasileiros de Endocrinologia e Metabologia, v. 56, p. 67-70, 2012.

- The rare intracellular *RET* mutation S891A in an apparently sporadic medullary thyroid carcinoma: case report and review of the literature. Blom CB, **Ceolin L**, Romitti M, Siqueira DR, Maia AL. Arquivos Brasileiros de Endocrinologia e Metabologia, v. 56, p. 586-591, 2012.

LISTA DE ABREVIATURAS E SIGLAS

ARE – Regiões do RNA ricas em adenina e uracila (AU-rich elements - ARE)

CLA – Líquen Amilóide Cutâneo

CMT – Carcinoma Medular de Tireóide

CMTF – Carcinoma Medular e Tireóide Familiar

GFL – Ligante da Família GNDF

GFRα – Receptores da Família GNDF

GNDF – Fator Neutrófico Derivado das células da Glía

HSCR – Doença de Hirschsprung

LD – Desequilíbrio de Ligação

MFE – Energia Mínima Livre

N_{DH} – Número de Hélices Duplas

NEM – Neoplasia Endócrina Múltipla Tipo

NEM 2 A – Neoplasia Endócrina Múltipla Tipo 2A

NEM 2B – Neoplasia Endócrina Múltipla Tipo 2B

NIH-3T3 – Linhagem celular de fibroblastos murinos

RET (REarranged during Transfection) – Proto-oncogene RET

SNP – Polimorfismos de Nucleotídeo Único

TK1 – Domínio intracelular da proteína RET rico em resíduos tirosino-quinase

TK2 – Domínio intracelular da proteína RET rico em resíduos tirosino-quinase

3′UTR – Região não traduzida 3'UTR

SUMÁRIO

RESUMO

Carcinoma medular da tiroide (CMT), tumor maligno originário de células C ou parafoliculares tireoidianas, representa cerca de 4% de todos os tumores malignos dessa glândula. O CMT ocorre principalmente na forma esporádica (75%), mas também pode ocorrer como parte de uma doença hereditária transmitida de forma autossômica dominante, com 100% de penetrância, chamada neoplasia endócrina múltipla tipo 2 (NEM 2). A síndrome NEM 2 é classificada em três subtipos clínicos distintos: NEM tipo 2A (NEM 2A); NEM tipo 2B (NEM 2B) e câncer medular de tireoide familiar (CMTF). O proto-oncogene *RET* (REarranged during Transfection) é o gene de susceptibilidade para CMT hereditário e mutações somáticas nesse gene são descritas em aproximadamente 50% dos casos CMT esporádicos. Polimorfismos de nucleotídeo único (SNPs) do proto-oncogene *RET* têm sido implicados na patogênese e progressão do carcinoma medular da tireoide. A presença da variante genética silenciosa S836S tem sido associada com o risco de desenvolver ou modificar o curso clínico do CMT. No entanto, o mecanismo exato com que esse polimorfismo exerce seu efeito ainda é pouco compreendido. Uma das hipóteses propostas sugere que outras variantes funcionais do *RET* possam estar em desequilíbrio de ligação (DL) com o polimorfismo S836S, sendo essas capazes de modular a expressão gênica. Na doença de Hirschsprung, a variante S836S está em DL com polimorfismos da região 3'UTR do *RET* e associada ao desenvolvimento da doença. Nesse estudo, nós investigamos a frequência dos polimorfismos rs76759170 e rs3026785 da região 3'UTR do proto-oncogene *RET* em pacientes com CMT e verificamos a presença de desequilíbrio de ligação entre essas variantes e o polimorfismo S836S. De forma interessante, observamos que as variantes 3'UTR podem afetar a estrutura e a flexibilidade do mRNA do *RET,* o que sugere um envolvimento funcional dessas variantes sobre a estrutura secundária do mRNA desse gene. Além disso, o haplótipo contendo os alelos polimórficos S836S e 3'UTR foi associado ao desenvolvimento de doença metastática em pacientes com CMT.

ABSTRACT

Medullary thyroid carcinoma (MTC), a malignant tumor originating in parafollicular C cells of the thyroid, represents about 4% of all thyroid cancers. MTC is mainly sporadic (75%), but may also be part of an inherited disorder transmitted as an autosomal dominant trait with 100% penetrance, referred as multiple endocrine neoplasia type 2 (MEN 2). The MEN 2 syndrome is classified into three distinct clinical subtypes: MEN type 2A (MEN 2A); MEN type 2B (MEN 2B) and familial MTC (FMTC). The *RET* (REarranged during Transfection) proto-oncogene is the susceptibility gene for hereditary MTC and somatic *RET* point mutations are described in approximately 50% of MTC cases. The *RET* single nucleotide polymorphisms (SNPs) have been implicated in the pathogenesis and progression of MTC. The presence of S836S neutral variant might modify disease susceptibility and clinical phenotype in MTC. However, the exact mechanism by which this polymorphism modulates the MTC pathogenesis is still poorly understood. One of the proposed mechanisms suggests that the S383S neutral variant might be in linkage disequilibrium (LD) with unknown functional variants; these might be modulating gene expression. In patients with Hirschsprung's disease, strong LD between S836S and *RET* 3'UTR variants has been reported. Here, we evaluated the frequency of rs76759170 and rs3026785 3'UTR polymorphisms in patients with MTC and observed strong LD between these variants and S836S polymorphism. Interestingly, we demonstrated that the 3'UTR variants may affect the *RET* mRNA structure and flexibility, supporting the hypothesis of a functional involvement of the 3'UTR variant allele on secondary structure of *RET* mRNA. Furthermore, the haplotype harboring these variants was associated with development of metastatic disease in individuals with MTC.

INTRODUÇÃO

O câncer de tireoide é considerado raro na maioria das populações mundiais, porém sua incidência tem aumentado continuamente nas ultimas três décadas (Pellegriti, et al. 2013). Em 2012, uma estimativa mundial apontou a ocorrência de aproximadamente de 300 mil novos casos dessa neoplasia, sendo 68 mil no sexo masculino e 230 mil no sexo feminino (Gobocan Estimated Cancer Incidence, Mortality and Prevalence Worldwide 2012). No Brasil, estima-se, para este ano, 1.150 novos casos de câncer de tireoide para o sexo masculino e 8.050 para o sexo feminino. Observa-se ainda que, na região sul do Brasil, o câncer de tireóide é a quarta neoplasia mais frequente em mulheres (INCA Estimativa 2014: Incidência de Câncer no Brasil).

O carcinoma medular de tireoide (CMT), uma neoplasia maligna das células C ou parafoliculares da tireoide, corresponde a aproximadamente 4% de todas as neoplasias malignas dessa glândula e é responsável por 13,5% dos óbitos relacionados a essa glândula (Davies and Welch 2006; Hundahl, et al. 1998). Em geral, a taxa de sobrevida dos pacientes com CMT é de 75% em 10 anos (Hundahl et al. 1998; Pelizzo, et al. 2007), entretanto, quando metástases a distancia estão presentes, a taxa de sobrevida diminui para 42% em 5 anos e 31% em 10 anos (Gilliland, et al. 1997; Hundahl et al. 1998). Em geral, aproximadamente 50% dos pacientes apresenta metástase local e 20% metástase a distância (Elisei, et al. 2008; Moura, et al. 2009; Scollo, et al. 2003).

O CMT pode ocorrer na forma esporádica, em cerca de 75% dos casos, ou como parte da síndrome de Neoplasia Endócrina Múltipla tipo 2 (NEM 2). A síndrome de NEM 2 inclui três formas clínicas distintas: NEM 2A, NEM 2B e CMT familiar (CMTF) . Nos casos de CMT familiar apenas a glândula tireóide é afetada. Pacientes com NEM 2A apresentam CMT, feocromocitoma e/ou hiperparatireoidismo; já os pacientes portadores de NEM 2B desenvolvem CMT, feocromocitoma, ganglioneuromas de trato gastrointestinal, neuromas de mucosa e/ou anormalidades esqueléticas (Kouvaraki, et al. 2005).

O proto-oncogene *RET* (REarranged during Transfection) é o gene de susceptibilidade para o CMT hereditário. Mutações germinativas de ganho de função na NEM 2A têm sido descritas nos exons 5, 8, 10, 11, 13, 14 e 15 do

RET (Ceolin et al 2012; Kouvaraki et al. 2005). No entanto, a maioria das famílias apresenta mutações em um dos cinco resíduos de cisteína do exon 10 (códons 609, 611, 618 e 620) ou do exon 11 (códon 634), no domínio extracelular do *RET* (Eng, et al. 1996; Ponder 1999). A presença de qualquer mutação no códon 634 tem sido associada com a presença de feocromocitoma e hiperparatireoidismo. Por outro lado, mutações nos códons 768 e 804 são associadas ao CMTF. As razões para as correlações genótipo-fenótipo ainda não foram completamente esclarecidas. Embora os diferentes níveis de ativação induzidos por diferentes mutações do *RET* possam explicar parcialmente essa questão, a variabilidade na apresentação clínica e agressividade do CMT observada em membros da mesma família sugerem a presença de outros modificadores genéticos no curso clínico desta doença (Machens, et al. 2001; Ponder 1999; Robledo, et al. 2003).

Um possível papel dos polimorfismos genéticos (*single nucleotide polymorphisms* - SNPs) do *RET* na patogênese do CMT ainda é uma questão em debate. As variantes genéticas mais estudadas no CMT são as seguintes: G691S (códon 691 do exon 11, GGT→AGT), L769L (códon 769 do exon 13, CTT→CTG), S836S (códon 836 do exon 14, AGC→AGT) e S904S (códon 904 do exon 15, TCC→TCG). Alguns estudos têm sugerido que os polimorfismos do *RET* podem interferir na apresentação clínica das síndromes hereditárias do CMT (Magalhães, et al. 2004; Rocha, et al. 2007). Robledo et al. demonstraram que duas dessas variantes do *RET* (G691S e S904S) podem alterar a idade de início do CMT em membros da mesma família (Robledo et al. 2003), embora estes resultados não tenham sido replicados posteriormente em uma grande amostra de população européia (Lesueur, et al. 2006). Também foi sugerido que o polimorfismo L769L pode ter contribuído para o início precoce do CMT em paciente com a mutação V804M (Magalhães et al. 2004). No ano de 2009, foi descrita associação dos polimorfismos IVS1-126G>T e [IVS8 82 A>G; 85_86insC] com o curso clínico do CMT hereditário em uma família portadora da mutação G533C do *RET* (Tamanaha, et al. 2009). No entanto, outros estudos não conseguiram demonstrar qualquer efeito dos polimorfismos do *RET* no curso natural do CMT hereditário (Fernandez, et al. 2006; Lesueur et al. 2006).

Com relação à forma esporádica da doença, estudos realizados em indivíduos europeus e norte-americanos descreveram uma maior prevalência do polimorfismo S836S em pacientes com CMT quando comparado ao grupo controle (Gimm, et al. 1999; Ruiz, et al. 2001). Mais tarde, uma associação entre os polimorfismos G691S e S904S e a ocorrência de CMT esporádico foi evidenciada em pacientes poloneses e ingleses (Cebrian, et al. 2005; Elisei, et al. 2004; Robledo et al. 2003). Entretanto, outros estudos não demonstraram diferenças quanto à frequência dos polimorfismos do *RET* entre pacientes com CMT esporádico e controles (Baumgartner-Parzer, et al. 2005; Berard, et al. 2004; Wohllk, et al. 2005).

Recentemente, um estudo do nosso grupo investigou a influência das variantes genéticas do *RET* (G691S, L769L, S836S e S904S) na apresentação clínica dos pacientes com CMT hereditário ou esporádico. Este trabalho demonstrou associação da variante S836S com diagnóstico precoce e maior risco para o desenvolvimento de doença metastática em pacientes com CMT hereditário ou esporádico (Siqueira, et al. 2010).

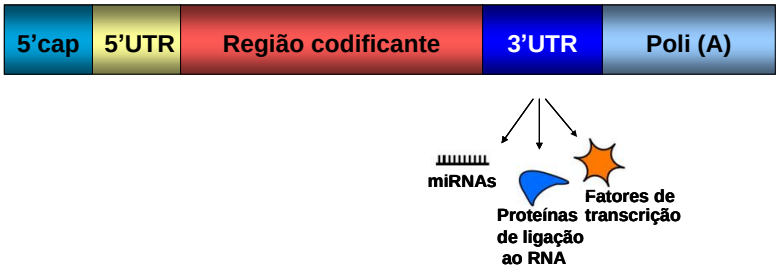
O mecanismo exato que explica o efeito dos polimorfismos genéticos na patogênese e/ou na evolução clínica do câncer não é conhecido; já que, na maioria das vezes, estas variantes não causam a substituição de aminoácidos e, dessa forma, não teriam um efeito cooperativo na dimerização da proteína RET ou na formação de um novo sítio de fosforilação. Uma das explicações possíveis para o efeito dessas variantes neutras é que elas poderiam modificar a estabilidade e/ou transcrição do RNA mensageiro. Outros mecanismos sugeridos seriam a criação de um sítio de *splicing* alternativo, gerando assim uma proteína alterada devido à troca de base na molécula do DNA, ou ainda os polimorfismos estudados poderiam estar em desequilíbrio de ligação com outra variante funcional ainda não conhecida (Ceolin, et al. 2011; Rocha et al. 2007; Tamanaha et al. 2009). Alguns autores investigaram esses mecanismos com relação à variante S836S; a análise dos níveis de RNA mensageiro do *RET* nos tumores de pacientes com CMT não demonstrou diferença quanto à presença ou não do polimorfismo S836S (Elisei et al. 2004). Além disso, estudos funcionais em células de CMT humano (células TT) não demonstraram

qualquer efeito do SNP S836S na estabilidade, splicing ou processamento do RNA (Griseri, et al. 2000).

Estudos em pacientes com doença de Hirschsprung (HSCR) - uma doença congênita associada em 30-50% dos casos a mutações inativadoras do gene *RET*- sugerem um papel do SNP S836S no desenvolvimento desta patologia. A análise de sete famílias italianas com HSCR evidenciou a presença de um raro haplótipo do *RET* associado a um efeito protetor. O estudo funcional desta região de 25.3 kb, que incluiu cinco polimorfismos do *RET:* A45A (G>A), L769L (T>G), S836S (C>T), S905S (C>G), IVS19+47C→TS- revelou que o haplótipo GGTCC correlacionou-se com a diminuição da quantidade de RNA mensageiro e aumento da isoforma RET51 (Griseri, et al. 2002).

Na tentativa de identificar variantes funcionais que possam estar em desequilíbrio de ligação com o SNP S836S, as variantes genéticas das regiões 5' e 3' do proto-oncogene *RET* também foram pesquisadas. Os SNPs -5pb (g.975820G>A) e -1pb (g.975824C>A) da região promotora do *RET* foram analisados em pacientes com HSCR, estando o primeiro em desequilíbrio de ligação com o SNP A45A (Fitze, et al. 2003). O haplótipo ACA (oriundo da combinação dos alelos polimóricos dos SNPs -5pb e A45A com o alelo *wild-type* -1pb) foi associado ao desenvolvimento de HSCR (Sancandi, et al. 2003; Tou, et al. 2011). Estudos funcionais em linfoblastos de pacientes com HSCR e controles evidenciaram menores níveis de expressão do RET em indivíduos com o haplótipo ACA. Contudo, nenhum destes polimorfismos isoladamente foi capaz de modificar os níveis de RNAm (Griseri, et al. 2005).

A presença de forte desequilíbrio de ligação na região 3' do *RET* (Carrasquillo, et al. 2002; Lantieri, et al. 2006) e da associação do SNP S836S com a expressão das diferentes isoformas do RET (Griseri et al. 2002), sugere que as variantes localizadas na região 3'UTR possam ter um efeito



Representação esquemática da estrutura de um mRNA eucarioto, demostrando a região 3'UTR e as principais moléculas que interagem com essa região.

funcional. Os polimorfismos g.128118G>A (rs76759170) e g.128496T>C (rs3026785) da região 3'UTR estão localizados em um fragmento rico em sequências AU, que são caracterizadas por sua importância na estabilidade do transcrito. Estudos funcionais em células de neuroblastoma humano sugeriram um efeito modificador do SNP g.128496T>C na estabilidade do RNAm. Porém, estudos semelhantes em células TT não reproduziram tais achados com este mesmo SNP (Griseri, et al. 2007).

É importante considerar que os possíveis efeitos dos polimorfismos em estudos funcionais podem depender do tipo célula empregada nos experimentos. Além disso, a presença e o tipo de mutação do *RET* nas células também podem determinar resultados diversos durante a análise do papel dos polimorfismos. Em células de neuroblastoma, o haplótipo contendo o polimorfismo g.128496T>C foi identificado como responsável pelas mudanças na expressão do gene, porém esse resultado não se reproduziu em células de CMT(Griseri et al. 2007; Griseri et al. 2002). Além disso, no estudo realizado por Elisei et al, que não identificou alteração na quantidade de RNAm, a pequena amostra de pacientes avaliada e falta de estratificação quanto a presença de mutação somática podem ser os motivos para os resultados negativos (Elisei et al. 2004).

Com base nesses conhecimentos, o objetivo desse estudo foi avaliar a frequência dos polimorfismos 3'UTR do proto-oncogene *RET* em pacientes com CMT e verificar a presença de desequilíbrio de ligação com a variante S836S.

Parte I

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

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Review

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. In particular, 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* (*RE arranged 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. In particular, we critically analyze the role of *RET* polymorphisms in the clinical presentation and prognosis of MTC.

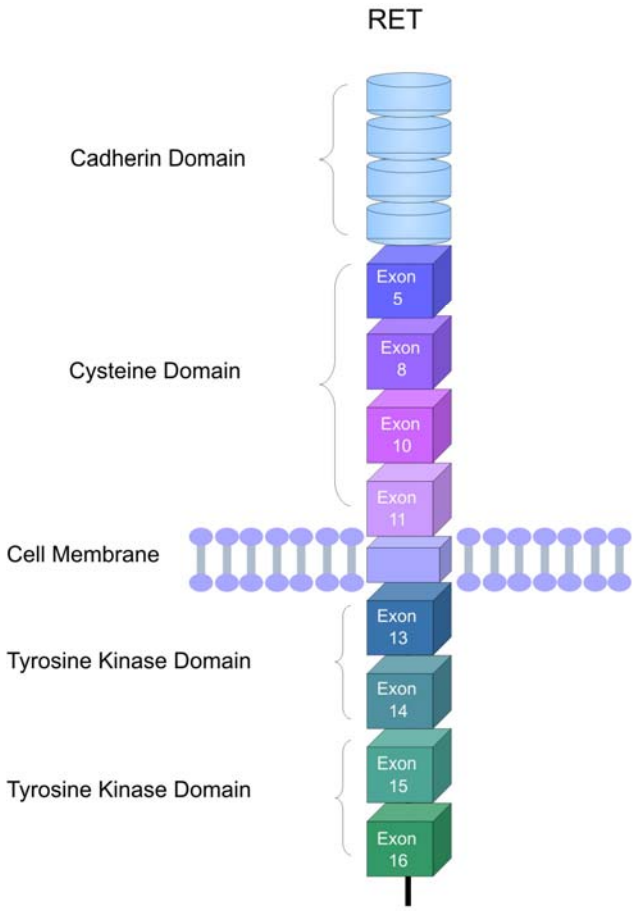
2. The *RET* Proto-Oncogene

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* (*RE arranged 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.



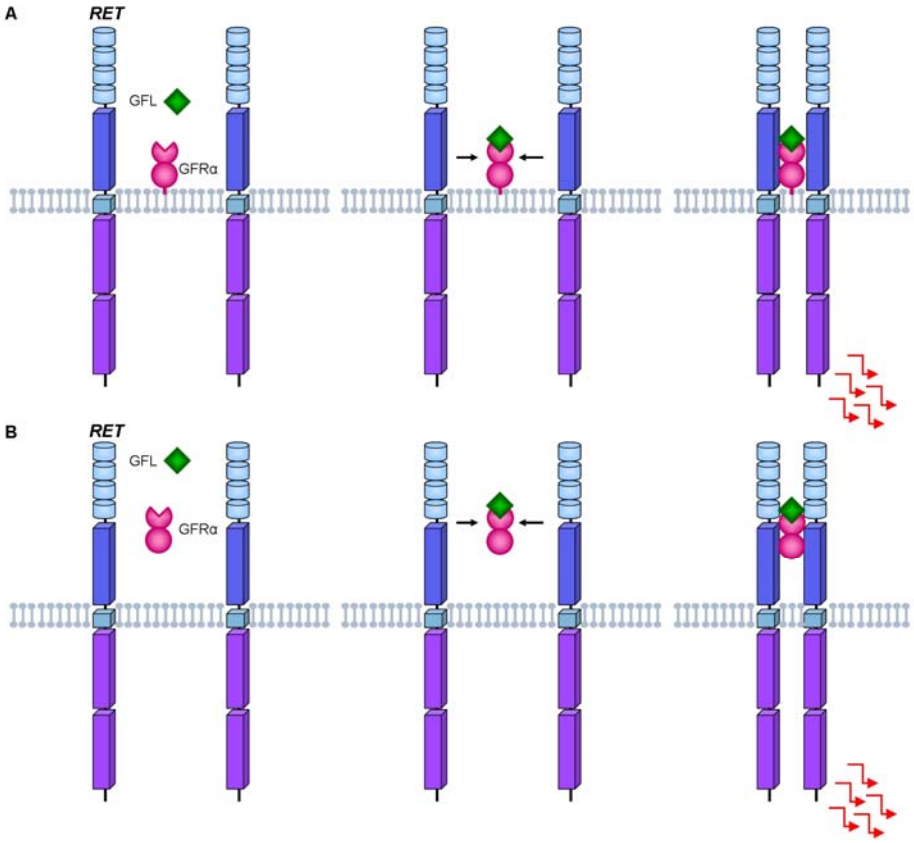
3. RET Protein Activation

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 GFRα 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].

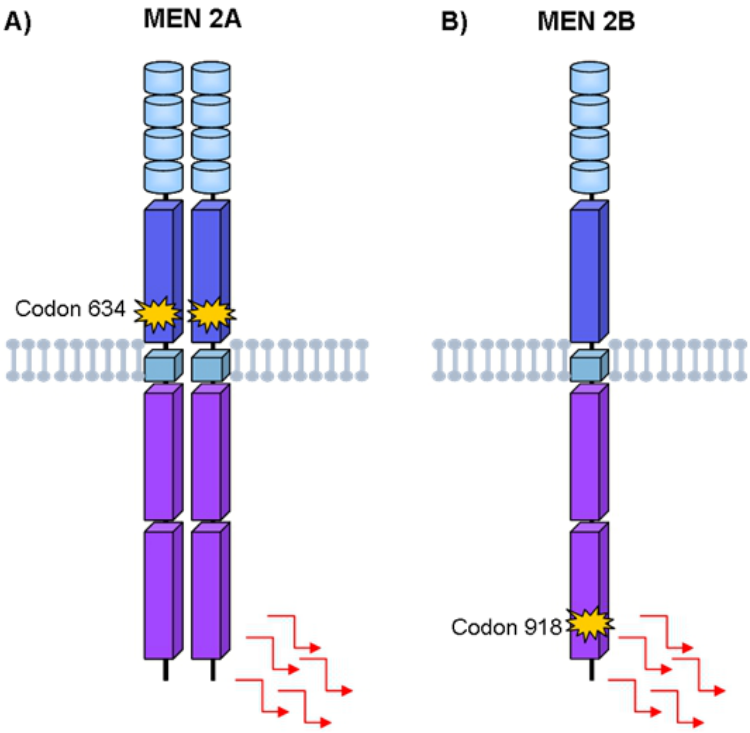
Figure 2. Mechanisms of ligand-mediated RET activation. (**A**) In the *cis* model RET activation: the glial cell line-derived neurotrophic factor (GDNF) family of ligands (GFL) binds to membrane glycosylphosphatidylinositol-anchored GDNF-family coreceptors (GFRα). The activation leads to dimerization 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].

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].

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 *RET* 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 IVS1−126 G > 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

Table 1. *Cont.*

RET variant	Author	Cases	Controls	*P*	Frequency (cases *vs.* controls)	Genotyping platform	Conclusion	Population
S836S (rs1800862)	Tamanah a (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
	Tamanah a (2009)	77 a	100	0.048	0; 4	RFLP	Underrepresented in G533C-carriers.	Brazilian
IVS1−126 G>T (rs2565206)	Tamanah a (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)	Tamanah a (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

Table 2. *Cont.*

RET variant	Author	Cases	Controls	*P*	Frequency (cases *vs.* controls)	Genotyping platform	Conclusion	Population
	Wiench (2001)	116 [b]	–	0.04 [b]	36; 15	sequencing	Associated with the presence of MTC in younger individuals	Polish
L769L (rs1800861)	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
	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
S836S (rs1800862)	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
	Wohllk (2005)	50	50	NS	6; 1	sequencing	N/A	Chilean
	Wohllk (2005)	50	50	NS	27; 28	sequencing	N/A	Chilean
S904S (rs1800863)	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 IVS1−126 G>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*, 152–161.
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*, 564–573.
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*, 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*, 319–342.
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*, 517–523.
6. Kebebew, E.; Ituarte, P.H.; Siperstein, A.E.; Duh, Q.; Clark, O.H. Medullary thyroid cancer. *Curr. Treat. Opt. Oncol.* **2000**, *1*, 359–367.
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*, 265–273.
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*, 5438–5443.
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*, 851–856.
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*, 905–909.
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.; *et al*. The relationship between specific *RET* proto-oncogene mutations and disease phenotype in multiple endocrine neoplasia type 2. International *RET* mutation consortium analysis. *J. Am. Med. Assoc.* **1996**, *276*, 1575–1579.
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*, 70–74.
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*, 458–460.

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*, 2167–2170.
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*, 249–257.
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*, 1619–1622.
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*, 953–963.
18. Takahashi, M.; Ritz, J.; Cooper, G.M. Activation of a novel human transforming gene, *RET*, by DNA rearrangement. *Cell* **1985**, *42*, 581–588.
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*, 1519–1521.
20. Takahashi, M.; Buma, Y.; Hiai, H. Isolation of *RET* proto-oncogene cDNA with an amino-terminal signal sequence. *Oncogene* **1989**, *4*, 805–806.
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*, 375–376.
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*, 2039–2045.
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*, 2925–2929.
24. Jing, S.; Wen, D.; Yu, Y.; Holst, P.L.; Luo, Y.; Fang, M.; Tamir, R.; Antonio, L.; Hu, Z.; Cupples, R.; *et al*. GDNF-induced activation of the RET protein tyrosine kinase is mediated by GDNFR-alpha, a novel receptor for GDNF. *Cell* **1996**, *85*, 1113–1124.
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*, 47898–47904.
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*, 4684–4696.
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*, 381–383.

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*, 2419–2427.
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. USA* **1994**, *91*, 1579–1583.
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 Chromosom. Cancer* **1993**, *6*, 166–177.
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*, 493–497.
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*, 343–346.
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*, 278–281.
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*, 229–236.
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*, 1104–1109.
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*, 2644–2649.
37. Donis-Keller, H. The *RET* proto-oncogene and cancer. *J. Intern. Med.* **1995**, *238*, 319–325.
38. Frank-Raue, K.; Hoppner, W.; Frilling, A.; Kotzerke, J.; Dralle, H.; Haase, R.; Mann, K.; Seif, F.; Kirchner, R.; Rendl, J.; *et al*. 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*, 1780–1783.
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*, 156–161.

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.; *et al*. 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*, 1035–1041.
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.; *et al*. Risk profiles and penetrance estimations in multiple endocrine neoplasia type 2A caused by germline *RET* mutations located in exon 10. *Hum. Mutat.* **2011**, *32*, 51–58.
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*, 3902–3904.
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*, 1213–1217.
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*, 393–397.
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*, 1–5.
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*, 3088–3090.
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*, 4176–4178.
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*, 2415–2419.
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*, 201–204.
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*, 509–513.
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.; *et al*. Medullary thyroid cancer: management guidelines of the American Thyroid Association. *Thyroid* **2009**, *19*, 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*, 60–69.
53. Elisei, R.; Cosci, B.; Romei, C.; Bottici, V.; Renzini, G.; Molinaro, E.; Agate, L.; Vivaldi, A.; Faviana, P.; Basolo, F.; *et al*. Prognostic significance of somatic *RET* oncogene mutations in sporadic medullary thyroid cancer: a 10-year follow-up study. *J. Clin. Endocrinol. Metab.* **2008**, *93*, 682–687.
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*, 1777–1783.
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*, 2063–2068.
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.; *et al*. Prognostic value of *RET* proto-oncogene point mutations in malignant and benign, sporadic phaeochromocytomas. *Int. J. Cancer* **1998**, *79*, 537–540.
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*, 1814–1817.
58. Wiench, M.; Wygoda, Z.; Gubala, E.; Wloch, J.; Lisowska, K.; Krassowski, J.; Scieglinska, D.; Fiszer-Kierzkowska, A.; Lange, D.; Kula, D.; *et al*. Estimation of risk of inherited medullary thyroid carcinoma in apparent sporadic patients. *J. Clin. Oncol.* **2001**, *19*, 1374–1380.
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 Chromosom. Cancer* **1995**, *12*, 209–212.
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*, 1369–1373.
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*, 3579–3584.
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*, 723–730.
63. Cebrian, A.; Lesueur, F.; Martin, S.; Leyland, J.; Ahmed, S.; Luccarini, C.; Smith, P.L.; Luben, R.; Whittaker, J.; *et al*. Polymorphisms in the initiators of *RET* (rearranged during transfection) signaling pathway and susceptibility to sporadic medullary thyroid carcinoma. *J. Clin. Endocrinol. Metab.* **2005**, *90*, 6268–6274.
64. Fugazzola, L.; Muzza, M.; Mian, C.; Cordella, D.; Barollo, S.; Alberti, L.; Cirello, V.; Dazzi, D.; Girelli, M.E.; Opocher, G.; *et al*. *RET* genotypes in sporadic medullary thyroid cancer: Studies in a large Italian series. *Clin. Endocrinol. (Oxf)* **2008**, *69*, 418–425.

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*, 1177–1180.
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*, 6232–6236.
67. Sharma, B.P.; Saranath, D. *RET* gene mutations and polymorphisms in medullary thyroid carcinomas in Indian patients. *J. Biosci.* **2011**, *36*, 603–611.
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*, 178–185.
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*, 848–852.
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*, 150–152.
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*, 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*, 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*, 721–724.
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*, 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*, 343–353.

Parte II

EFFECT OF 3′UTR *RET* VARIANTS ON THE SECONDARY STRUCTURE OF *RET* mRNA IN MEDULLARY THYROID CARCINOMA

EFFECT OF 3′UTR *RET* VARIANTS ON THE SECONDARY STRUCTURE OF RET mRNA IN MEDULLARY THYROID CARCINOMA

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Running Title: 3′UTR *RET* polymorphisms in linkage disequilibrium with S836S variant.

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Abstract

The presence of the *RET* S836S variant has been associated with early onset and increased risk for metastatic disease in medullary thyroid carcinoma (MTC). However, the mechanism as SNPs modulate the MTC pathogenesis is still open to discuss. One of the proposed mechanisms suggests that the S383S neutral variant might be in linkage disequilibrium (LD) with unknown functional variants. Interestingly, strong LD between S836S and *RET* 3'UTR variants has been reported in patients with Hirschsprung's disease. **Objective**: To evaluate the frequency of the *RET* 3'UTR variants (rs76759170 and rs3026785) in MTC patients and to determine whether these variants are in LD with S836S polymorphism. **Methods**: Our sample comprised 282 patients with sporadic or hereditary MTC. The *RET* variants S836S and 3'UTR (rs76759170 and rs3026785) were genotyped using Custom TaqMan Genotyping Assays. Haplotypes were inferred using the phase 2.1 program. *RET* mRNA structure and flexibility was assessed by Vienna Package and TfReg software. **Results:** Of the 282 patients analyzed, 155 (55.0%) presented hereditary and 127 (45.0%) had the sporadic form of disease. The mean age of diagnosis was 36.7±19 years and 56.3% were women. The minor allele frequencies of *RET* polymorphic variants were as follows: S836S(4.8%), rs76759170(4.9%) and rs3026785(4.9%). We observed a strong LD between 3'UTR variants (|D'|= -1, r^2= 1) and between these variants and S836S polymorphism (|D'|= -1, r^2= 0,989). Interestingly, the haplotype carrying the S836S and 3'UTR *RET* variants presented a greater number of double helices sections and lower levels of minimal free energy when compared to the wild-type haplotype. Patients harboring the haplotype with S836S and 3'UTR variants presented more aggressive disease as compared those harboring the wild-type haplotype. **Conclusion**: Our results demonstrated that the neutral *RET* polymorphism S836S is in LD with *RET* 3'UTR variants and these variants may affect the secondary structure of *RET* mRNA, supporting the hypothesis of a functional involvement of the 3'UTR variant allele on *RET* mRNA structure and flexibility.

INTRODUCTION

Medullary thyroid carcinoma (MTC), a malignant tumor originating in parafollicular C cells of the thyroid, represents about 4% of all thyroid cancers (Davies and Welch 2006). MTC is mainly sporadic (75%), but may also be part of an inherited disorder transmitted as an autosomal dominant trait with 100% penetrance, referred to as multiple endocrine neoplasia type 2 (MEN 2). The MEN 2 syndrome is classified into three distinct clinical subtypes: MEN type 2A (MEN 2A): characterized by the presence of MTC, pheochromocytoma (PHEO), and hyperparathyroidism (HPT); MEN type 2B (MEN 2B): that includes MTC, PHEO, ganglioneuromatosis, and marfanoid habitus, and familial MTC (FMTC): characterized by MTC as the only feature of the disease (Clark, et al. 2005; Pelizzo, et al. 2007). The *RET* (REarranged during Transfection) proto-oncogene is the susceptibility gene for hereditary MTC and somatic *RET* point mutations in exon 16 (M918T) are described in approximately 50% of MTC (Marsh, et al. 1996; Mulligan, et al. 1994; Siqueira, et al. 2010).

Several studies have investigated the relationship between the presence of single nucleotide polymorphisms (SNPs) in the *RET* gene and susceptibility or progression of hereditary or sporadic MTC. The *RET* neutral variants L769L, S836S, and S904S influence the clinical presentation and disease outcome in MTC patiens (Ceolin, et al. 2012a; Gimm, et al. 1999; Siqueira et al. 2010; Sromek, et al. 2010; Wiench, et al. 2001). Particularly, the S836S variant (codon 836 of exon 14, SerAGC/SerAGT) has been found in higher frequency as compared to control group, in sporadic MTC patients (Gimm et al. 1999). This variant also has been associated with two-to-three-fold increase in the risk of MTC in the Spanish population (Ruiz, et al. 2001), early onset of MTC and increased risk for metastatic disease (Siqueira et al. 2010). These results are in agreement with a recent meta-analysis that demonstrated an association of sporadic MTC susceptibility with S836S polymorphism (Figlioli, et al. 2012). In hereditary MTC, the S836S was over-represented in G533C-carriers (Tamanaha, et al. 2009) and associated with early onset of MTC and increased risk for metastatic disease (Siqueira et al. 2010). Recently, the presence of multiple *RET* risk alleles has been associated with increased estimated risk for MTC development and aggressiveness, indicating that these variants could be

acting in an additive manner on disease pathogenesis (Ceolin et al. 2012a; Siqueira, et al. 2014).

Nevertheless, the mechanism as SNPs modulate the MTC pathogenesis is still open to discuss (Ceolin, et al. 2012b; Figlioli et al. 2012). One of the proposed mechanisms suggests that the S383S neutral variant might be in linkage disequilibrium (LD) with an unknown functional variant. The unknown variant may be modulating the *RET* oncogene expression to affect the structural stability of the mRNA (Fernandez, et al. 2006; Gimm et al. 1999; Tamanaha et al. 2009).

Loss-of-function *RET* mutations account for approximately 50% of familial and 7–35% of sporadic Hirschsprung's disease (HSCR) patients (Attie, et al. 1994; Lyonnet, et al. 1994). Furthermore, several *RET* polymorphisms and haplotypes have been described as underrepresented in HSCR patients, and could act as low susceptibility loci and modify the phenotype of HSCR (Fitze, et al. 2003; Griseri, et al. 2005; Lantieri, et al. 2006). Particularly, the haplotype including the rarer S836S polymorphic allele has shown a low penetrant protective effect against the disease (Fitze, et al. 2002; Griseri, et al. 2002). Interestingly, Griseri et al 2007 report a strong LD among S836S variant and 3'untranslated region (3'URT) SNPs (rs76759170, G>A and rs3026785, T>C). This study suggests that 3'UTR variants can have a functional effect on the *RET* expression (Griseri, et al. 2007).

Here, we evaluated the presence of linkage disequilibrium between S836S and 3'UTR *RET* variants in MTC patients and assessed whether the 3'UTR variants could play a role on *RET* mRNA structural stability.

MATERIAL AND METHODS

Patients

Patients with a diagnosis of 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 *RET* mutation screening in Brazil, and therefore, patients referred to us by other Brazilian centers were also invited to participate. All patients and/or their legal

representatives provided written informed consent for the study 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 (HIRS) 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, USA, reference range <10 pg/ml and, after January 2004, Immulite 2000, Diagnostic Products Corporation, Los Angeles, CA, USA; reference value (VR) male <12.0 pg/ml and female <6.0 pg/ml)), plasma parathyroid hormone (PTH; Immulite 2000 Intact PTH, Diagnostic Products), urinary fractionated metanephrines (HPLC), and, whenever indicated, diagnostic imaging investigation (cervical ultrasonography, thorax and abdominal computed tomography (CT)). Selected patients were submitted to whole-body metaiodobenzylguanidine scintigraphy to rule out PHEO and/or distant metastasis.

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 TNM classification (O'Sullivan and Shah 2003).

Our initial sample comprised 297 patients, 168 with hereditary MTC and 129 with the sporadic form of disease. Fifteen of these patients were excluded, either because they were awaiting surgery or not enough material was available for polymorphism analysis.

Genotyping assay

RET variants L769L (rs1800861, codon 769, exon 13, LeuCTT/LeuCTG), S836S (rs1800862; codon 836, exon 14, SerAGC/SerAGT), S904S (rs1800863, codon 904, exon 15, SerTCC/SerTCG) and 3'UTR (rs3026785, T>C and rs76759170, G>A) were analyzed in DNA extracted from peripheral blood leukocytes by standardized procedures. Genotype analysis was performed using Human Custom TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA), as described previously by (Ceolin et al. 2012a; Siqueira et al. 2014). Primer and probe sequences used for genotyping the *RET* 3'UTR variants were, rs3026785: 50-CACGTAAATGCAGAAGTTACTAAGTATTAAGTATTACT-30 (forward primer), 50-AGGAACATGATCTGGTTTAATGACCTTT-30 (reverse primer), VIC-50-TCTGTCAGTTATTAAAATT-30, FAM-50-TGTCAGTTACTAAAATT-30; rs75759170: 50-ACACGTAACCTGGCTCTAATTTGG-30 (forward primer), 50-CTGCATTTAGTAAGACTATCATTAAGCATATCTGA-30 (reverse primer), VIC-50-CACAGTGTATCTGAAAAA-30, FAM-50-CACAGTGTATTTGAAAAA-30.

The reactions were conducted in a 96-well plate, in a total 5 ml reaction volume using 2-10ng genomic DNA, TaqMan Genotyping Master Mix 1X (Applied Biosystems), and Custom TaqMan Genotyping Assay 1X. The plates were then positioned in a real-time PCR thermal cycler (7500 Fast Real PCR System; Applied Biosystems) and heated for 10 min at 95 8C, followed by 45 cycles of 95 ºC for 15s and 62 ºC for 1min. Fluorescence data files from each plate were analyzed using automated allele calling software (SDS 2.1; Applied Biosystems).

Haplotype Construction and Linkage Disequilibrium Analysis

The haplotypes were constructed based on the combination of allelic variants and their frequencies were inferred using the phase 2.1 program, which implements a Bayesian statistical method (Stephens, et al. 2001). We also used the phase 2.1 program to compare the distributions of different *RET* haplotypes between MTC patients through permutation analyses of 1000 random replicates (Stephens et al. 2001). Among all pairs of biallelic loci, we examined widely used measures of linkage disequilibrium (LD), Lewontin's D' lD'l and r^2 (Hedrick 1987).

Thermodynamic Simulations Methodology

The accuracy of computer predictions of RNA secondary structure from sequence data and free energy parameters is about 70% and the predictions are easier and more accurate on small molecules (the number of structures grows exponentially with the sequence length) (Jaeger, et al. 1989; Mathews 2004; Wuchty, et al. 1999).

In this study, to minimize the experimental limitations and perform a more robust analysis, we evaluate *RET* optimal and suboptimal mRNA structures. We believe it is important to evaluate both structures because there could be short or long-lived intermediate states of the RNA sequence while it executes the folding process that will ultimately lead it to the minimal free energy (MFE) state. In other words, the RNA may stay longer in suboptimal folded structures than in the final MFE structure.

For the bioinformatics analyses we used the haplotypes inferred by Phase program (Table 3). Additionally, we also used the haplotype carrying only the S836S variant (TTCGT). This haplotype does not exist in our population; however, we decided to use it to compare with the GTCAC haplotype.

The preliminary analysis treated both, wild type (TCCGT_WT haplotype) and polymorphic sequences (GCCGT, TCGGT and GTCAC haplotypes), with the RNAfold algorithm provided by the Vienna Package. This algorithm provides the optimal RNA secondary structure and its associated MFE for each input sequence (Lorenz, et al. 2011).

The Vienna Package (Lorenz et al. 2011) program RNAsubopt with default parameters was used to generate suboptimal secondary structures of all haplotypes. Since the number of possible suboptimal structures for each sequence is exceedingly large we randomly selected a subset of those using the -p option. In this analysis we used subsets of 2900 structures for each sequence. The resulting dot-bracket files were evaluated as follows: 1) by counting the number of continuous base-pairs in double helices of at least 6 bp (number of double helices sections - N_{DH}); 2) by using TfReg to evaluate the total (Weber 2013) melting index of the double helices; 3) by calculating the resulting Gibbs free energy with RNAeval from the Vienna Package (Lorenz et al. 2011).

First we perform these calculations for the wildtype sequence and obtain average numbers of base-pairs, total melting index and Gibbs free energy. Then we compare each suboptimal structure of the polymorphic haplotype to those averages and count how many times they present greater thermodynamic stability. This gives us a probability which represents how likely it is that a polymorphic haplotype may result into a more stable structure than the wild type sequence.

The NCBI Reference Sequence accession number for the *RET* gene was: NM_020975.4 (mRNA).

Statistical analysis

Results are expressed as mean±S.D. or median (IQ 25–75) unless otherwise specified. Hardy–Weinberg equilibrium for each polymorphism was assessed by χ^2 tests. Baseline characteristics were compared using χ^2 tests or Fisher's exact test for qualitative variables. Quantitative variables were compared between groups using Student's t-test or Kruskal–Wallis tests. 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. The Statistical Package for the Social Sciences 18.0 (SPSS Inc., Chicago, IL, USA) was used, and $P<0.05$ was considered as statistically significant.

RESULTS

RET variants in medullary thyroid carcinoma patients

The clinical and oncological features of the study subjects are summarized in Table 1. The median age at diagnosis was 36.7±9 years and the percentage of women was 56.3%. The ethnic background was 95% Caucasian. Of the 282 patients analyzed, 155 (55.0%) presented hereditary MTC and 127 (45%) presented sporadic form of disease. The median basal serum calcitonin and CEA levels at diagnosis were 269.5 (30.8-1206.3)pg/ml and 13 (2.7-47.8)ng/ml, respectively. The median tumor size was 3.4 (2.2-4.0) cm. One

hundred and ten patients (46.8%) presented lymph node metastasis and 17.6% presented distant metastasis at diagnosis, respectively. The minor allele frequencies observed were as follows: S836S (4.8%) and 3'UTR *RET* variants rs76759170 (4.9%) and rs3026785 (4.9%) (Table 2).

Haplotype Construction and Linkage Disequilibrium Analysis

We used a Bayesian statistical method to estimate the frequency of different haplotypes produced by the combination of the L769L(rs1800861), S836S(rs1800862), S904S(rs1800863) and 3'UTR(rs3026785 and rs76759170) polymorphisms in MTC patients.

A total of four haplotypes were inferred in our study population. The haplotype frequencies were shown in Table 3. Interestingly, we observed a strong LD between 3'UTR variants (|D'|= -1, r^2= 1). Likewise, the 3'UTR polymorphisms are also in a strong LD with the S836S variant (|D'|= -1, r^2= 0,989).

Bioinformatics Analysis

Next, we evaluated the effect of the *RET* haplotypes on the optimal and suboptimal mRNA structures. Our purpose was to evaluate the effect of these variants on the whole structure, not only in part. Thus, we opted to analyze the full mRNA sequence (5.6kb). For the bioinformatics analyses we used all haplotypes inferred by Phase program (Table 3). These analyses included only synonymous polymorphisms.

As shown in Figure 1, bioinformatics analyses have demonstrated different RNA secondary structure predictions for WT (TCCGT), S836S (TTCGT) and 3'UTR (GTCAC) haplotypes.

The 3'UTR haplotype presented greater number of N_{DH} in both optimal and suboptimal structures, when compared with the WT haplotype (Figure 2B and 2D, respectively). In agreement, bioinformatics analysis showed differences in minimal free energy (MFE) for the WT and 3'UTR haplotypes in optimal and suboptimal structures (Figure 2A and 2C, respectively). We also assessed whether the 3'UTR have an additional effect on the *RET* mutations M918T and C634Y, the most frequent mutation at codon 634 in our sample. As shown in

Figure 3, the 3'UTR haplotype polymorphisms affect the N*DH* and MFE structures even in the presence of the *RET* mutations (Figure 3).

RET genotypes and disease presentation

Due to the distinct clinical features of the sporadic and hereditary disease, we performed the analysis separately.

Sporadic Medullary Thyroid Carcinoma

Table 1 shows the clinical and oncological features of the 127 patients with sporadic MTC patients. The mean age at diagnosis was 48±15.5 years and 53.5% was women. The frequency of lymph node and distant metastasis were 57.4 and 24.5%, respectively.

The minor allele frequencies observed for the *RET* variants were follows: S836S (5.5%), rs76759170 (5.6%) and rs3026785 (5.6%) (Table 2). Fourteen patients (11%) were heterozygous for 3'UTR polymorphic allele. Individuals harboring the 3'UTR haplotype had higher serum calcitonin level (1747.5(939.2-7444) *vs* 579(120-2582.5) pg/mL, P=0.028), CEA level (585(144.7-4353) *vs* 28.3(6.5-113) ng/mL, P=0.005) and larger tumor size (3.4(2.2-4.0) *vs* 2.3(1.5-3.3), P=0.050). The 3'UTR *RET* haplotype was associated with metastatic disease as compared to WT haplotype (100 *vs* 52.9%, P=0.003 and 69.2 *vs* 25.0%, P=0.003) (Table 4). Accordingly, Kaplan–Meier estimates of cumulative lymph node and distant metastasis yielded distinct curves for patients with or without the 3'UTR haplotype (P<0.001, Figure 4), further demonstrating that metastatic disease occurred earlier in those individuals harboring the 3'UTR variants.

Hereditary Medullary Thyroid Carcinoma

A total of 155 patients presented the hereditary form of the disease. The clinical and oncological features of MEN2 families are shows in Table 5. Of the 35 independent families with MEN2 analyzed, 19 families were classified as MEN2A, 3 as MEN2A associated with cutaneous lichen amyloidosis (CLA), 1 as MEN2A associated with Hirschsprung's disease, 9 as MEN2B and 3 as FMTC. All but three MEN2A kindred had a RET codon 634 mutation (exon 11), the

most prevalent mutation (91% of cases). All MEN2B individuals presented mutation at codon 918 (exon 16), resulting in the substitution of a methionine residue by threonine (M918T).

The mean age at diagnosis was 27.2±16.7 years and 58.6% was women (Table 1). The frequency of lymph node and distant metastasis were 36.7 and 11.3%, respectively (Table 1). The frequency of *RET* 3'UTR polymorphic variants (rs76759170 and rs3026785) was 4.2% (Table 2).There were no differences in age at diagnosis, serum calcitonin and CEA levels, tumor size, PHEO and lymph node or distant metastasis between individuals with or without 3'UTR polymorphic alleles (P>0.05). However, the frequency of HPT was higher in individuals harboring the 3'UTR haplotype (15 *vs* 45%, P=0.035) (Table 4). Interestingly, Kaplan–Meier estimates of cumulative lymph node yielded distinct curves for patients with or without the 3'UTR haplotype (P=0.041, Figure 5). However, no differences were observed for distant metastasis (P=0.164, Figure 5).

DISCUSSION

In the present study, we have demonstrated that the neutral *RET* polymorphism S836S is in linkage disequilibrium with *RET* 3'UTR variants. Interestingly, the haplotype carrying the S836S and 3'UTR *RET* variants presented a greater N_{DH} and lower levels of MFE as compared to the wild-type haplotype. Furthermore, the haplotype harboring these variants are associated with development of metastatic disease at a younger age in individuals with hereditary or sporadic MTC.

Based on the overrepresentation of the *RET* polymorphisms in individuals with hereditary or sporadic MTC, a role for these genetic variants in the MTC pathogenesis has been suggested. Nevertheless, the results on the effect of *RET* polymorphic variants in the development or progression of MTC is still controversial (Ceolin et al. 2012b; Figlioli et al. 2012; Machens, et al. 2012). 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). More recently, the S836S variant was associated with early onset of MTC and

increased risk for metastatic disease in sporadic and hereditary form of disease (Siqueira et al. 2010). Nevertheless, several other studies failed to demonstrate any effect of *RET* variants on risk of development or on the natural course of MTC (Berard, et al. 2004; Sharma and Saranath 2011; Wohllk, et al. 2005).

The molecular mechanism by which *RET* polymorphisms affect the development and evolution of MTC are still not properly understood. One of the proposed mechanisms suggests that polymorphisms could influence the *RET* mRNA levels. However, quantitative studies in MTC tumor samples show no difference in *RET* expression in patients with or without the G691S/S904S, L769L, and S836S polymorphisms (Elisei, et al. 2004). Another hypothesis suggests that bases exchange in the DNA molecule could create a new alternative splicing site, leading to the synthesis of a truncated protein, erroneous ligand binding, micro-RNA binding, change of structure and mRNA stability as well as a number of copies (Borrego, et al. 1999). However, the study performed by Griseri et al. showed that the S836S(C>T) polymorphic variant failed to affect DNA–protein binding, transcript stability, or RNA splicing and editing of the *RET* gene (Griseri, et al. 2000). An alternative way to explain the role of *RET* polymorphisms in the clinical course of MTC proposes that these neutral variants can be in LD with an unknown functional variant (Borrego, et al. 2003). Griseri et al 2007 report a strong LD between S836S polymorphism and 3'UTR variants in HSCR disease. This study suggests that 3'UTR variants play a role in the posttranscriptional control of a subset of *RET* transcripts (Griseri et al. 2007).

Here, we have demonstrated that the S836S polymorphism is in linkage disequilibrium with *RET* 3'UTR variants in MTC patients. The 3'UTR gene region is emerging as fundamental and effective in regulating gene expression at posttranscriptional levels (pre-mRNA processing, mRNA stability and translational regulation) (Moore 2005). Several sequence elements that may be involved in mRNA regulation exist in the 3′UTR region, including regions rich in adenine and uridine elements - AU-rich elements (ARE). The ARE mRNAs are regulated by RNA-binding proteins that can selectively bind to the ARE and promote their mRNA stability or degradation (Lopez de Silanes, et al. 2007). Griseri et al found that rs3026785 is located next to the AUUUA sequence. This study showed that single nucleotide substitution of 3′UTR may influence the

secondary structure of *RET* mRNA and that 3'UTR allele lowers *RET* mRNA degradation in human neuroblastoma cells, ultimately leading to an increase of transcription product and probably an increase in the amount of total *RET* protein at the cell membrane, however, the same effect was no found in transfected MTC cells (Griseri et al. 2007; Griseri et al. 2002).

Corroborating these findings, *in silico* analysis performed in our study showed that 3'UTR haplotype (GTCAC) could influence the stability of the *RET* mRNA. We can observe that this haplotype presents highest N_{DH} and lower levels of MFE, suggesting that *RET* mRNA carrying the 3'UTR haplotype provides the most rigid and thermodynamically stable mRNA structure, when compared with the others. Thus, our data allow us to suggest that the 3'UTR haplotype may affect the secondary structure of *RET* mRNA, supporting the hypothesis of a functional involvement of the 3'UTR variant allele in *RET* mRNA stability.

CONCLUSION

Our results demonstrate linkage disequilibrium between S836S and *RET* 3'UTR genetic variants. The *RET* mRNA carrying the 3'UTR haplotype presents higher structural and thermodynamic stability when compared to mRNA carrying the WT haplotype, supporting the hypothesis of a functional involvement of the 3'UTR variant allele in *RET* mRNA stability. Furthermore, patients harboring 3'UTR haplotype presents more aggressive disease as compared to those harboring the WT haplotype.

REFERENCES

Attie T, Edery P, Lyonnet S, Nihoul-Fekete C & Munnich A 1994 [Identification of mutation of RET proto-oncogene in Hirschsprung disease]. *C R Seances Soc Biol Fil* **188** 499-504.

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, Saez ME, Ruiz A, Gimm O, Lopez-Alonso M, Antinolo G & Eng C 1999 Specific polymorphisms in the RET proto-oncogene are over-represented in patients with Hirschsprung disease and may represent loci modifying phenotypic expression. *J Med Genet* **36** 771-774.

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.

Ceolin L, Siqueira DR, Ferreira CV, Romitti M, Maia SC, Leiria L, Crispim D, Ashton-Prolla P & Maia AL 2012a Additive effect of RET polymorphisms on sporadic medullary thyroid carcinoma susceptibility and tumor aggressiveness. *Eur J Endocrinol* **166** 847-854.

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

Clark JR, Fridman TR, Odell MJ, Brierley J, Walfish PG & Freeman JL 2005 Prognostic variables and calcitonin in medullary thyroid cancer. *Laryngoscope* **115** 1445-1450.

Davies L & Welch HG 2006 Increasing incidence of thyroid cancer in the United States, 1973-2002. *JAMA* **295** 2164-2167.

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.

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

Figlioli G, Landi S, Romei C, Elisei R & Gemignani F 2012 Medullary thyroid carcinoma (MTC) and RET proto-oncogene: mutation spectrum in the familial cases and a meta-analysis of studies on the sporadic form. *Mutat Res* **752** 36-44.

Fitze G, Appelt H, Konig IR, Gorgens H, Stein U, Walther W, Gossen M, Schreiber M, Ziegler A, Roesner D, et al. 2003 Functional haplotypes of the

RET proto-oncogene promoter are associated with Hirschsprung disease (HSCR). *Hum Mol Genet* **12** 3207-3214.

Fitze G, Cramer J, Ziegler A, Schierz M, Schreiber M, Kuhlisch E, Roesner D & Schackert HK 2002 Association between c135G/A genotype and RET proto-oncogene germline mutations and phenotype of Hirschsprung's disease. *Lancet* **359** 1200-1205.

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, Bachetti T, Puppo F, Lantieri F, Ravazzolo R, Devoto M & Ceccherini I 2005 A common haplotype at the 5' end of the RET proto-oncogene, overrepresented in Hirschsprung patients, is associated with reduced gene expression. *Hum Mutat* **25** 189-195.

Griseri P, Lantieri F, Puppo F, Bachetti T, Di Duca M, Ravazzolo R & Ceccherini I 2007 A common variant located in the 3'UTR of the RET gene is associated with protection from Hirschsprung disease. *Hum Mutat* **28** 168-176.

Griseri P, Pesce B, Patrone G, Osinga J, Puppo F, Sancandi M, Hofstra R, Romeo G, Ravazzolo R, Devoto M, et al. 2002 A rare haplotype of the RET proto-oncogene is a risk-modifying allele in hirschsprung disease. *Am J Hum Genet* **71** 969-974.

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.

Hedrick PW 1987 Gametic disequilibrium in cats: epistasis is an unlikely cause. *J Hered* **78** 204.

Jaeger JA, Turner DH & Zuker M 1989 Improved predictions of secondary structures for RNA. *Proc Natl Acad Sci U S A* **86** 7706-7710.

Lantieri F, Griseri P, Puppo F, Campus R, Martucciello G, Ravazzolo R, Devoto M & Ceccherini I 2006 Haplotypes of the human RET proto-oncogene associated with Hirschsprung disease in the Italian population derive from a single ancestral combination of alleles. *Ann Hum Genet* **70** 12-26.

Lopez de Silanes I, Quesada MP & Esteller M 2007 Aberrant regulation of messenger RNA 3'-untranslated region in human cancer. *Cell Oncol* **29** 1-17.

Lorenz R, Bernhart SH, Honer Zu Siederdissen C, Tafer H, Flamm C, Stadler PF & Hofacker IL 2011 ViennaRNA Package 2.0. *Algorithms Mol Biol* **6** 26.

Lyonnet S, Edery P, Mulligan LM, Pelet A, Dow E, Abel L, Holder S, Nihoul-Fekete C, Ponder BA & Munnich A 1994 [Mutations of RET proto-oncogene in Hirschsprung disease]. *C R Acad Sci III* **317** 358-362.

Machens A, Frank-Raue K, Lorenz K, Rondot S, Raue F & Dralle H 2012 Clinical relevance of RET variants G691S, L769L, S836S and S904S to sporadic medullary thyroid cancer. *Clin Endocrinol (Oxf)* **76** 691-697.

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.

Mathews DH 2004 Using an RNA secondary structure partition function to determine confidence in base pairs predicted by free energy minimization. *RNA* **10** 1178-1190.

Moore MJ 2005 From birth to death: the complex lives of eukaryotic mRNAs. *Science* **309** 1514-1518.

Mulligan LM, Eng C, Healey CS, Clayton D, Kwok JB, Gardner E, Ponder MA, Frilling A, Jackson CE, Lehnert H, et al. 1994 Specific mutations of the RET proto-oncogene are related to disease phenotype in MEN 2A and FMTC. *Nat Genet* **6** 70-74.

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

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.

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.

Sharma BP & Saranath D 2011 RET gene mutations and polymorphisms in medullary thyroid carcinomas in Indian patients. *J Biosci* **36** 603-611.

Siqueira DR, Ceolin L, Ferreira CV, Romitti M, Maia SC, Maciel LM & Maia AL 2014 Role of RET genetic variants in MEN2-associated pheochromocytoma. *Eur J Endocrinol* **170** 821-828.

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.

Sromek M, Czetwertynska M, Skasko E, Zielinska J, Czapczak D & Steffen J 2010 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* **21** 178-185.

Stephens M, Smith NJ & Donnelly P 2001 A new statistical method for haplotype reconstruction from population data. *Am J Hum Genet* **68** 978-989.

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.

Weber G 2013 TfReg: calculating DNA and RNA melting temperatures and opening profiles with mesoscopic models. *Bioinformatics* **29** 1345-1347.

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, Soto E, Bravo M & Becker P 2005 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* **133**.

Wuchty S, Fontana W, Hofacker IL & Schuster P 1999 Complete suboptimal folding of RNA and the stability of secondary structures. *Biopolymers* **49** 145-165.

Tables

Table 1. The clinical and oncological features of medullary thyroid carcinoma patients

	Total	Sporadic MTC	Hereditary MTC
Total patients (%)[a]	282	127(45.0)	155(55.0)
Sex female (%)	160(56.3)	68(53.5)	91(58.6)
Age at Diagnosis (yr)[b]	36.7±19	48±15.5[1]	27.2±16.7[7]
Calcitonin (pg/ml)[c]	269.5(30.8-1206.3)	759(163.5-2777.5)[2]	103(17.8-882.6)[8]
CEA (ng/ml)[c]	13(2.7-47.8)	36(7.2-123)[3]	6.3(2-30.1)[9]
Tumor Size (cm)[c]	2.3(1.3-3.2)	2.4(1.5-3.5)[4]	1.5(0.7-2.9)[10]
N1 (%)	116(48.9)	67(58.3)[5]	49(40.2)[11]
M1 (%)	46(20.9)	32(30.5)[6]	14(12.2)[12]

CEA, carcinoembryonic antigen; N1, lymph node metastasis; M1, distant metastasis.

[a]Fifteen of the 297 patients were excluded, either because they were awaiting surgery or not enough material was available for polymorphism analysis (15 patients).

[b]Data expressed as mean±S.D.

[c]Data expressed as median (IQ 25–75).

[1] Dados avaliados para 118 pacientes;

[2] Dados avaliados para 60 pacientes;

[3] Dados avaliados para 34 pacientes;

[4] Dados avaliados para 82 pacientes;

[5] Dados avaliados para 115 pacientes;

[6] Dados avaliados para 105 pacientes;

[7] Dados avaliados para 142 pacientes;

[8] Dados avaliados para 106 pacientes;

[9] Dados avaliados para 55 pacientes;

[10] Dados avaliados para 30 pacientes;

[11] Dados avaliados para 123 pacientes;

[12] Dados avaliados para 116 pacientes.

Table 2. *RET* minor allele frequency polymorphisms in medullary thyroid carcinoma patients

RET Polymorphisms	Allele variation	Total (n=282)	Sporadic MTC (n=127)	Hereditary MTC (n=155)
S836S (%)	C>T	4.8	5.5	4.2
rs76759170 (%)	G>A	4.9	5.6	4.2
rs3026785 (%)	T>C	4.9	5.6	4.2

Table 3. Haplotypes inferred by the Phase Program

	Presence/absence of					Haplotype (%)[a]	
Haplotypes	L769L	S836S	S904S	rs76759170	rs3026785	Sporadic	Hereditary
TCCGT (WT)						0.53	0.61
GCCGT	X					0.20	0.20
TCGGT			X			0.22	0.15
GTCAC	X	X		X	X	0.05	0.04

Haplotype frequencies was calculated by the Phase 2.0 program using permutation test (1000 replications). For simplified representation, the Phase program shows the haplotypes by the presence of the wild-type or risk allele .
[a] Frequencies calculated by the Phase 2.0 program based on the number of chromosomes.

Table 4. Clinical and oncological features of medullary thyroid carcinoma patients according to the presence of polymorphic haplotype

	Sporadic MTC		
	WT	GTCAC	*P*
Total patients (%)	113	14	
Sex female (%)	63(55.8)	5(35.7)	0.129[c]
Age at Diagnosis (yr)[a]	48.8±16	42±11	0.095[d]
Calcitonin (pg/ml)[b]	579(120-2582.5)	1747.5(939.2-7444)	0.028[d]
CEA (ng/ml)[b]	28.3(6.5-113)	585(144.7-4353)	0.005[d]
Size Tumor (cm)[b]	2.3(1.5-3.3)	3.4(2.2-4.0)	0.050[d]
PHEO (%)	-	-	-
HPT (%)	-	-	-
N1 (%)	54(52.9)	13(100)	0.003[c]
M1 (%)	23(25.0)	9(69.2)	0.003[c]

	Hereditary MTC		
	WT	GTCAC	*P*
Total patients (%)	142	13	
Sex female (%)	85(60)	6(46.2)	0.505
Age at Diagnosis (yr)[a]	27±17	23.2±11	0.428[d]
Calcitonin (pg/ml)[b]	90(16.5-882.8)	410.9(19.0-1066)	0.577[d]
CEA (ng/ml)[b]	6.3(2.0-33.0)	12.5(1.08-)	0.652[d]
Size Tumor (cm)[b]	1.5(0.7-2.9)	1.7[e]	0.933[d]
PHEO (%)	37(32.5)	5(41.7)	0.748
HPT (%)	17(15)	5(45.5)	0.035
N1 (%)	43(39)	6(50)	0.543[c]
M1 (%)	12(11.7)	2(18.2)	0.885[c]

PHEO, pheochromocytoma; HPT, hyperparathyroidism; N1, lymph node metastasis; M1, distant metastasis; WT, wild-type; GTCAC, haplotype harboring S836S and 3'UTR *RET* variants.

[a]Data expressed as mean±S.D.

[b]Data expressed as median (IQ 25–75).

[c]Variables were compared using the Yates' X^2-test or Fisher's exact test.

[d]Variables were compared using the Mann–Whitney U test.

[e]Data about only one patient.

Table 5. Clinical presentation and *RET* germline mutations in hereditary medullary thyroid carcinoma paitients.

Phenotype	Nº of *RET* families	*RET* mutation	Mutation ATA risk level	MTC	PHEO	HPT
MEN2A	9	C634Y	C	59	16	12
	5	C634R	C	14	6	2
	1	C634W	C	3	2	2
	1	C634S	C	1	1	-
	1	C620R	B	3	1	1
	1	C618R	B	5	2	0
	1	C618F	B	2	0	0
MEN2A+CLA	1	C634Y	C	28	6	6
	1	C634W	C	4	2	0
	1	C634R	C	2	0	0
MEN2A+HIRS	1	C618R	B	1	1	0
MEN2B	9	M918T	D	13	5	0
FMTC	1	E768D	A	10	0	0
	1	S891A	A	9	0	0
	1	L790F	A	1	0	0

MTC, hereditary medullary carcinoma; PHEO, pheochromocytoma; HPT, hyperparathyroidism

Figures

Figure 1

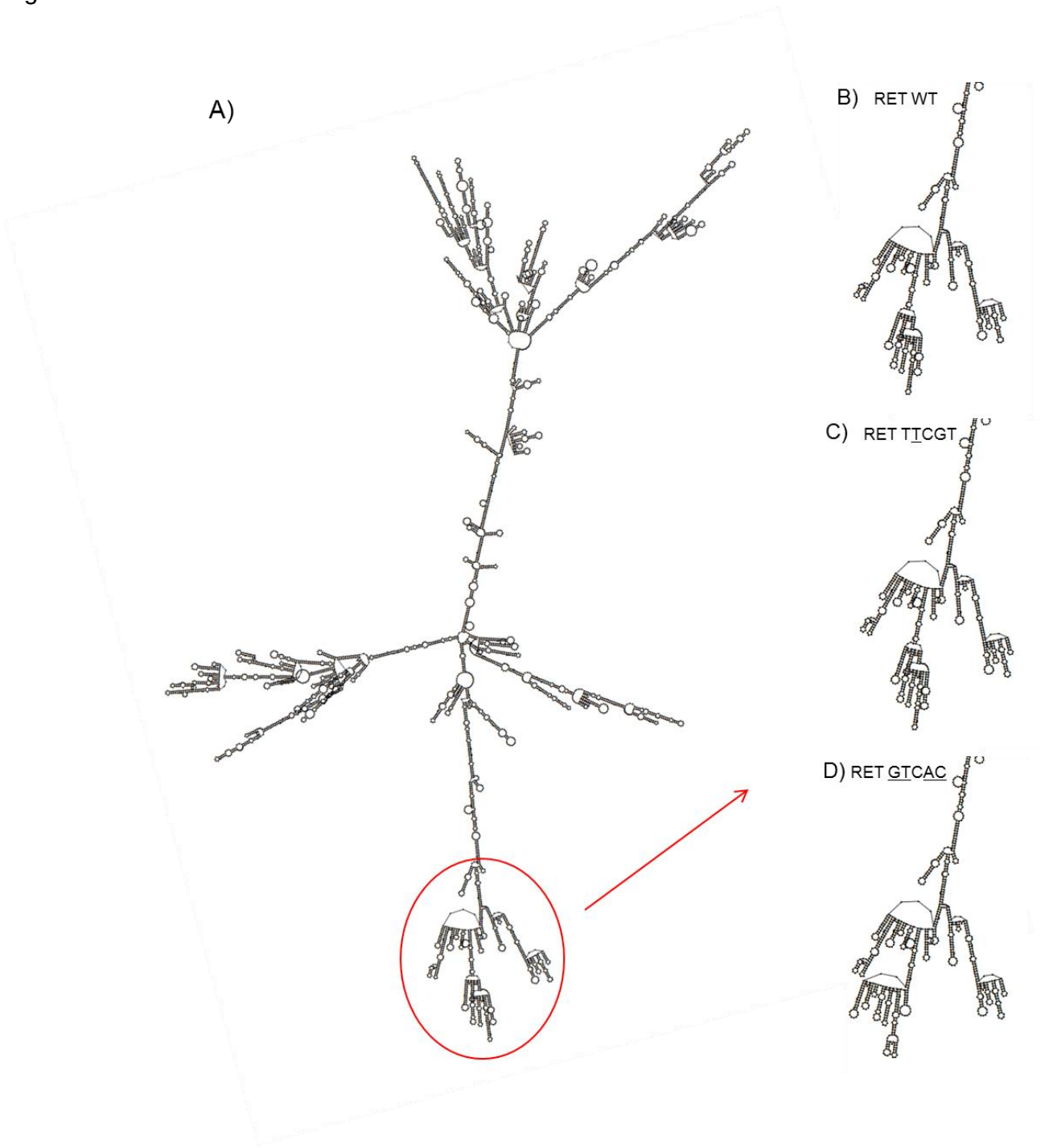

Figure 1. The figure shows the optimal mRNA secondary structure of the *RET* haplotypes. A) Total mRNA secondary structure of the *RET* wild-type (WT) haplotype. B) Part of mRNA secondary structure of the *RET* WT haplotype. C) Part of mRNA secondary structure of the *RET* S836S haplotype (TTCGT). D) Part of mRNA secondary structure of the *RET* 3'UTR haplotype (GTCAC). Haplotypes generated by RNAfold program (Vienna Package).

Figure 2

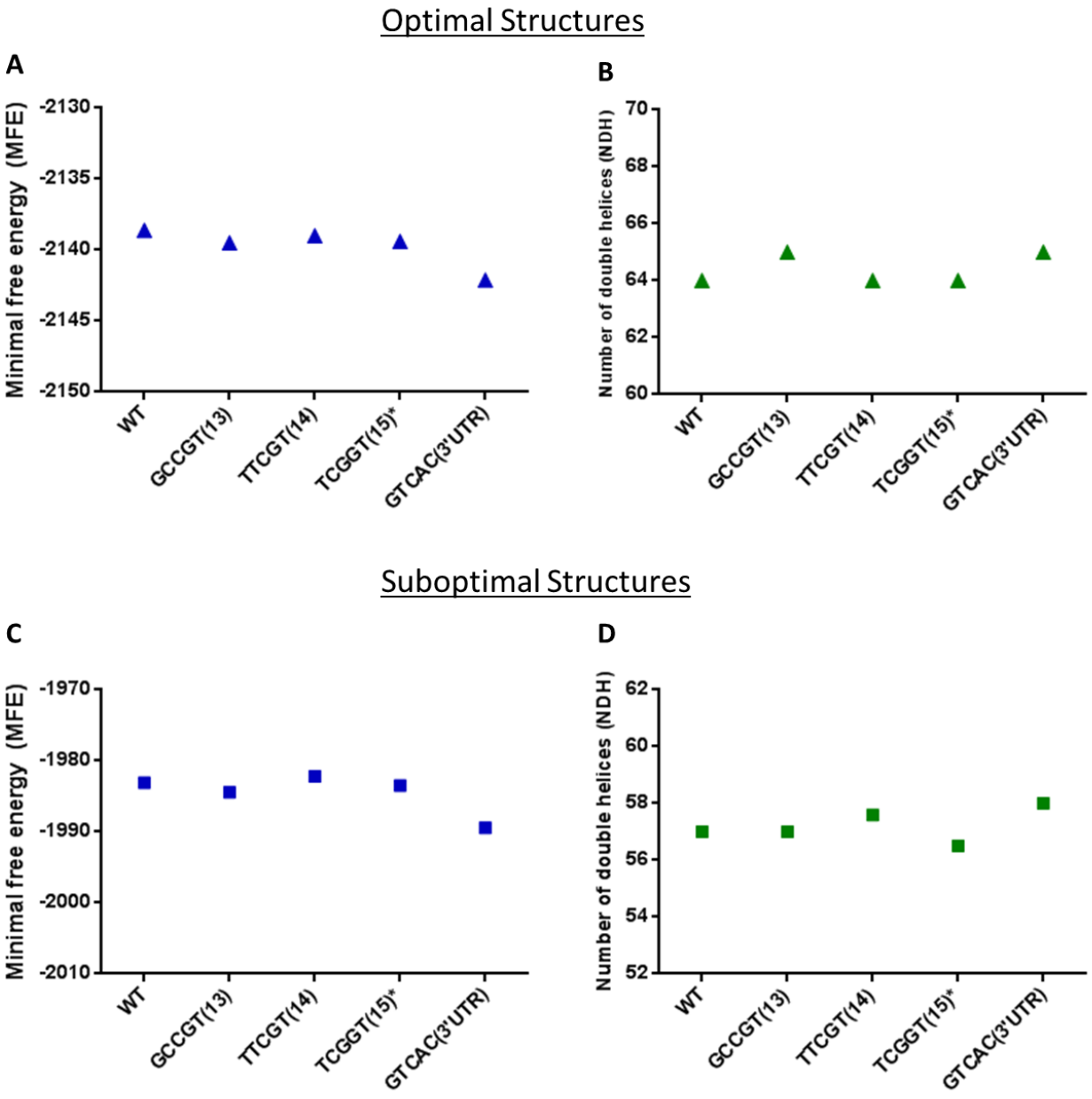

Figure 2. The minimal free energy (MFE) and number of double helices (N_{DH}) were available in both, optimal and suboptimal structures. The variant fragment carrying the S836S and 3'UTR variants (GTCAC haplotype) presented greater N_{DH} (B,D) and lower levels of MFE (A,C) when compared to wild-type haplotype (WT, TTCCGT), this fact happens in both, optimal and suboptimal structures. *These analyze included only synonymous polymorphisms. The G691S variant was excluded by promote amino acid substitution, thus affecting directly the protein structure.

Figure 3

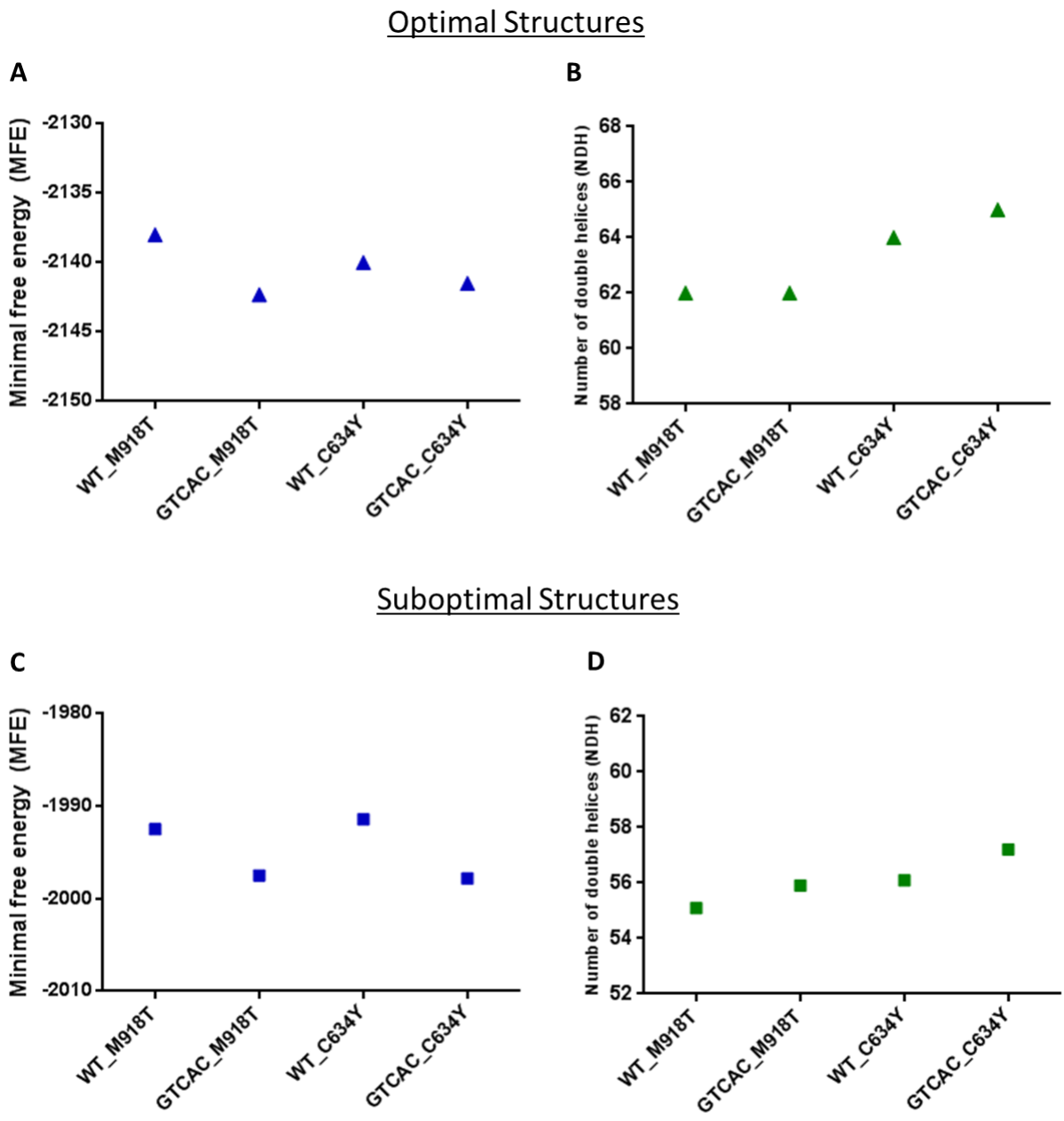

Figure 3. Minimal free energy (MFE) and number of double helices (N_{DH}) in optimal and suboptimal mutated sequences. The mutated sequences carrying the S836S and 3'UTR variants (GTCAC haplotype) presented greater N_{DH} (B,D) when compared to mutated sequences carrying the WT sequence. The mutated sequences carrying the S836S and 3'UTR variants (GTCAC haplotype) also presented lower levels of MFE in optimal (A) and suboptimal (B) structures when compared to mutated sequences carrying the WT haplotype.

Figure 4

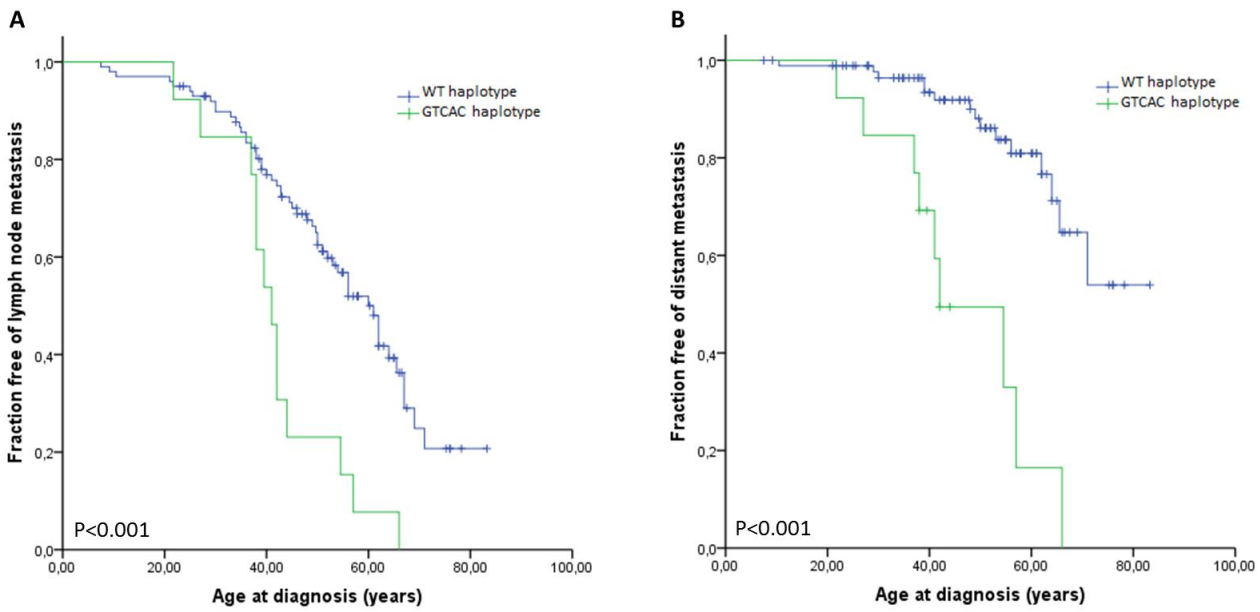

Figure 4. Kaplan–Meier estimates the proportion of sporadic MTC patients (n=127) with lymph node (A) or distant metastasis (B). The log rank test was used to compare curves.

Figure 5

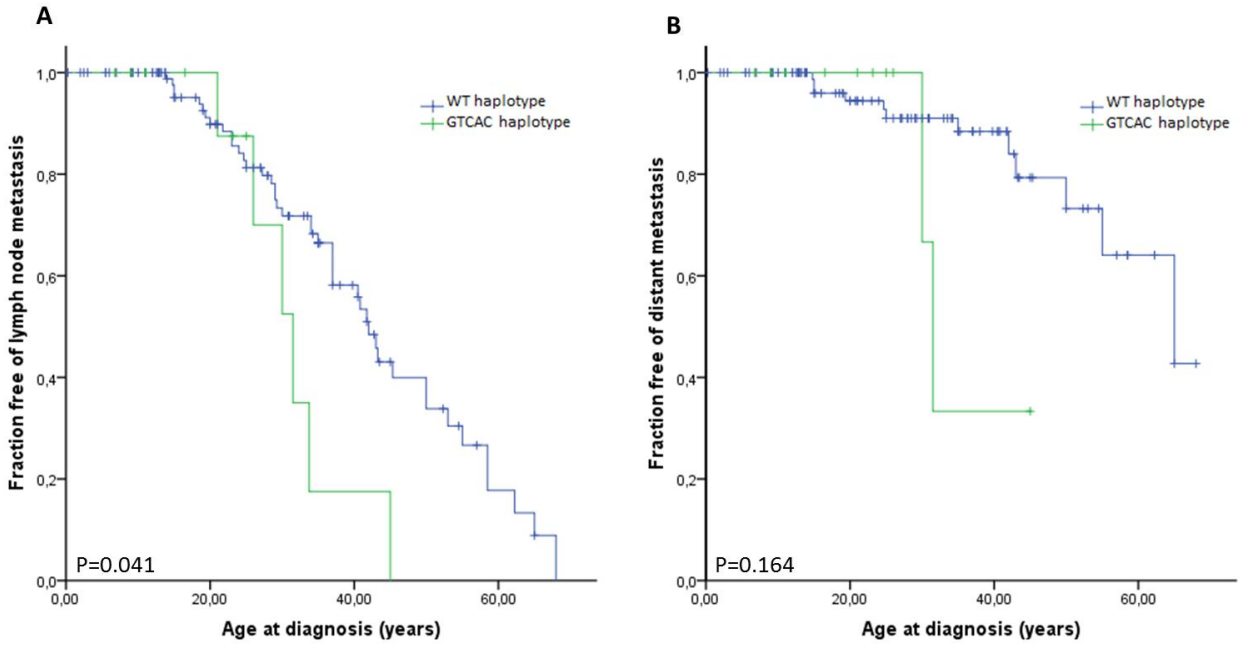

Figure 5. Kaplan–Meier estimates the proportion of hereditary MTC patients (n=155) with lymph node (A) or distant metastasis (B). The log rank test was used to compare curves.

CONCLUSÃO

Os nossos resultados demonstraram que o polimorfismo S836S do proto-oncogene *RET* está em desequilíbrio de ligação com variantes localizadas na região 3'UTR desse gene. Além disso, observamos que a sequência de mRNA do *RET* portadora do haplótipo S836S e 3'UTR (GTCAC) apresenta maior estabilidade estrutural e termidinâmica quando comparado a sequência selvagem (haplótipo TCCGT). Dessa forma, sugerimos que estas variantes podem afetar a estrutura secundária do mRNA do gene *RET*, influenciando assim a estabilidade dos seus transcritos.

REFERÊNCIAS

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.

Carrasquillo MM, McCallion AS, Puffenberger EG, Kashuk CS, Nouri N & Chakravarti A 2002 Genome-wide association study and mouse model identify interaction between RET and EDNRB pathways in Hirschsprung disease. *Nat Genet* **32** 237-244.

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.

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* **12** 221-39.

Davies L & Welch HG 2006 Increasing incidence of thyroid cancer in the United States, 1973-2002. *JAMA* **295** 2164-2167.

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 2006 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.

Fitze G, Appelt H, Konig IR, Gorgens H, Stein U, Walther W, Gossen M, Schreiber M, Ziegler A, Roesner D, et al. 2003 Functional haplotypes of the RET proto-

oncogene promoter are associated with Hirschsprung disease (HSCR). *Hum Mol Genet* **12** 3207-3214.

Gilliland FD, Hunt WC, Morris DM & Key CR 1997 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* **79** 564-573.

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.

Gobocan Estimated Cancer Incidence, Mortality and Prevalence Worldwide 2012 http://globocan.iarc.fr/Pages/online.aspx.

Griseri P, Bachetti T, Puppo F, Lantieri F, Ravazzolo R, Devoto M & Ceccherini I 2005 A common haplotype at the 5' end of the RET proto-oncogene, overrepresented in Hirschsprung patients, is associated with reduced gene expression. *Hum Mutat* **25** 189-195.

Griseri P, Lantieri F, Puppo F, Bachetti T, Di Duca M, Ravazzolo R & Ceccherini I 2007 A common variant located in the 3'UTR of the RET gene is associated with protection from Hirschsprung disease. *Hum Mutat* **28** 168-176.

Griseri P, Pesce B, Patrone G, Osinga J, Puppo F, Sancandi M, Hofstra R, Romeo G, Ravazzolo R, Devoto M, et al. 2002 A rare haplotype of the RET proto-oncogene is a risk-modifying allele in hirschsprung disease. *Am J Hum Genet* **71** 969-974.

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.

Hundahl SA, Fleming ID, Fremgen AM & Menck HR 1998 A National Cancer Data Base report on 53,856 cases of thyroid carcinoma treated in the U.S., 1985-1995 [see commetns]. *Cancer* **83** 2638-2648.

INCA Estimativa 2014: Incidência de Câncer no Brasil http://www1.inca.gov.br/vigilancia/incidencia.html.

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.

Lantieri F, Griseri P, Puppo F, Campus R, Martucciello G, Ravazzolo R, Devoto M & Ceccherini I 2006 Haplotypes of the human RET proto-oncogene associated with Hirschsprung disease in the Italian population derive from a single ancestral combination of alleles. *Ann Hum Genet* **70** 12-26.

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.

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.

Magalhães 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.

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.

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.

Pellegriti G, Frasca F, Regalbuto C, Squatrito S & Vigneri R 2013 Worldwide increasing incidence of thyroid cancer: update on epidemiology and risk factors. *J Cancer Epidemiol* **2013** 965212.

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

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, Magalhães PR, Maia AL & Maciel LZ 2007 Polimorfismos Genéticos: Implicações na Patogênese do Carcinoma Medular de Tireóide. *Arq Bras Endocrinol Metab* **5** 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.

Sancandi M, Griseri P, Pesce B, Patrone G, Puppo F, Lerone M, Martucciello G, Romeo G, Ravazzolo R, Devoto M, et al. 2003 Single nucleotide polymorphic alleles in the 5' region of the RET proto-oncogene define a risk haplotype in Hirschsprung's disease. *J Med Genet* **40** 714-718.

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.

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.

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.

Tou J, Wang L, Liu L, Wang Y, Zhong R, Duan S, Liu W, Xiong Q, Gu Q, Yang H, et al. 2011 Genetic variants in RET and risk of Hirschsprung's disease in Southeastern Chinese: a haplotype-based analysis. *BMC Med Genet* **12** 32.

Wohllk N, Soto E, Bravo M & Becker P 2005 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* **133**.