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Dissertação
Mestrado em Biologia Evolutiva e do Desenvolvimento
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Dissertação orientada por:
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Abstract

Thyroid carcinoma is the most frequent endocrine malignancy.

Medullary thyroid carcinoma (MTC) is one of the thyroid tumors and represents about 5 to 8% of all cases. It arises from C cells (parafollicular cells), in contrast with the other thyroid tumors that arise from follicular cells.

MTC can occur either in a sporadic or hereditary form, being the latter associated to multiple endocrine neoplastic type 2 (MEN2) and familial MTC (FMTC).

RET (rearrangement during transfection) encodes a membrane receptor tyrosine kinase that, when mutated, can originate MTC.

The aim of this project was to evaluate, through *in vitro* functional experimental approaches, the oncogenic potential of two newly identified RET germinal variants detected in two MTC patients from our institution: RET C515T (RET missense alteration TGC→TGG at codon 515 - Cys515Trp) and RET T636M (RET missense alteration ACG→ATG at codon 636 - Thr636Met.

By expressing the novel RET variants in non-transformed NIH 3T3 mouse fibroblasts, and comparing their effect with wild-type RET, we were able to observe an increase of cell growth and proliferation, loss of contact inhibition and an increase in cell migration rate. All these are common characteristics of tumoral cells, indicating that these new RET variants hold some relevant transforming potential. This transforming potential is, nevertheless, low when compared to that of RET MEN2A-causing mutation, C634R, which is consistent with the mild phenotype, observed in the patients of this study, characterized by late onset and low aggressiveness.

The functional characterization of these two new variants not only contributes to the understanding of the molecular mechanisms behind the development of MTC in these families, but also provides useful information to optimize the diagnostic and clinical management of hereditary MTC.

Keywords: RET, MTC, thyroid, carcinoma and mutation

Resumo

Após um quarto de século de grandes avanços, a investigação na área da oncologia tornou-se rica e complexa, mostrando assim que o cancro é uma doença que envolve mudanças dinâmicas no genoma. Muitas evidências indicam que a tumorigénese em humanos é um processo com várias etapas, que reflete não só as alterações no genoma, mas também que levam a uma transformação progressiva de células normais em células derivadas altamente malignas.

Carcinoma é qualquer tumor maligno que provenha de células epiteliais, e que pode originar metástases. Existem inúmeros tipos de carcinoma, sendo os carcinomas endócrinos um deles, abrangendo uma enorme variedade, tanto a nível do fenótipo como do genótipo.

Os carcinomas da tiróide são o tipo de carcinomas endócrinos mais frequentes, e podem ser divididos em dois grandes grupos: os carcinomas da tiróide que provêm de células foliculares e os carcinomas da tiróide que provêm de células parafoliculares.

Os carcinomas medulares da tiróide (CMT) representam cerca de 5 a 8% dos carcinomas da tiróide. Estes desenvolvem-se a partir de células C (células parafoliculares), enquanto os outros tipos de tumores desenvolvem-se a partir das células foliculares.

Os carcinomas medulares podem ocorrer de forma esporádica (cerca de 75% do total dos casos) ou hereditária (cerca de 25%), sendo esta última associada a síndrome de neoplasia endócrina múltipla de tipo 2 (NEM2), subdividida em NEM2A e NEM2B, e CMT familiar (CMTF).

Os carcinomas medulares da tiróide possuem uma enorme variedade, que podem ir de tumores com bom prognóstico, a carcinomas altamentes agressivos, com uma elevada taxa de mortalidade.

Mutações no proto oncogene RET (Rearranjado durante a transfeção) estão na origem do desenvolvimento do carcinoma medular da tiróide. Este proto oncogene codifica para um recetor membranar com atividade de tirosina-cinase e está envolvido em várias vias de sinalização que controlam processos como a sobrevivência e proliferação celular, migração e angiogénese, entre outros.

Os casos de CMTF estão geralmente associados a mutações pontuais a nível germinal no gene RET que podem ser mutações que afetam a região extracelular ou o domínio de tirosina-cinase deste recetor.

Nos casos onde não existe qualquer mutação na proteína RET, as cisteínas extracelulares formam ligações de bissulfito intramoleculares, estabilizando o recetor na sua conformação monomérica. O domínio das cisteínas é altamente conservado e tem um papel importante na manutenção da estrutura secundária e terciária do domínio extracelular do RET, através destas ligações de bissulfito. Quando ocorre uma mutação que leva à perda de um resíduo de cisteína esta torna-se livre para formar uma ligação intermolecular com outro receptor mutado, ocorrendo assim a dimerização e fosforilação da proteína RET, tornando-a ativa, mesmo na ausência de ligandos.

Assim, mutações ativadoras neste gene conduzem a uma ativação não-regulada destas vias, levando assim ao surgimento do fenótipo tumoral.

Este trabalho tem como objectivo a realização de estudos funcionais *in vitro* para duas variantes germinais do proto-oncogene RET, não descritas na literatura, associadas a dois casos distintos de carcinoma medular da tiróide (referidos como paciente A e paciente B), tentando assim avaliar seu o grau de patogenicidade.

A paciente A, sem evidência clínica na família de síndrome NEM2A/2B ou CMTF, foi submetida, aos 79 anos, a tiroidectomia total devido a um CMT unifocal. O teste genético ao gene RET identificou uma variante heterozigótica no codão 636 (ACG→ATG) no exão 11, que resulta na substituição do aminoácido treonina por metionina na região extracelular da proteína RET, designada por RET T636M. Apenas um membro da família desta paciente foi testado, a filha de 50 anos que apresentava bócio multinodular, tendo-se verificado ser negativa para esta variante.

A paciente B tinha 60 anos à data do diagnóstico, tendo sido submetida a tiroidectomia total devido a CMT multifocal. O teste genético ao gene RET revelou uma variante germinal presente em homozigotia no exão 8, codão 515 (TGC→TGG), que leva à substituição de uma cisteína por um triptofano no domínio extracelular da proteína RET, designada por RET C515T.

O facto dos pais desta paciente serem primos em primeiro grau levou provavelmente a esta variante se apresentar em homozigotia. Esta variante foi

também encontrada, apesar de em heterozigotia, em oito de dez familiares estudados. Nenhum destes apresentou qualquer sintoma sugestivo de CMT ou NEM2, exceto uma prima que também veio a desenvolver CMT aos 63 anos.

O potencial oncogénico de uma variante pode ser estimado através de diversos ensaios funcionais *in vitro*, avaliando-se assim sua capacidade de transformação induzida pela mutação comparativamente com o controlo normal, sem mutação. Para estes estudos tem-se em conta as características específicas das células tumorais, que as distinguem das células normais, não transformadas. Para tal, utiliza-se normalmente a linha celular de fibroblastos de ratinho NIH 3T3, comercialmente disponível, sendo analisado o efeito da expressão exógena do péptido mutante nestas células.

Antes de se dar inicio aos ensaios funcionais, efetuou-se a análise funcional *in silico* para prever o efeito patogénico de cada variante.

Os ensaios funcionais incluíram o ensaio de formação de focos, migração, crescimento e ciclo celular. A expressão exógena do gene RET nas células transfetadas foi monitorizada e quantificada pela técnica de western-blott.

O resultado destes estudos funcionais revelou que as novas variantes do gene RET, RET C515T e RET T636M, em comparação com o efeito induzido pelo RET selvagem, levavam um aumento do crescimento e proliferação celular, à perda de inibição por contacto e um aumento da migração celular. Todas estas características são comuns às células tumorais, levantando-se assim a hipótese de que estas novas variantes podem possuir um potencial oncogénico relevante. O potencial transformante destas novas variantes foi, no entanto, inferior ao observado para a mutação RET C634R, causadora do fenótipo NEM2A e utilizada como controlo positivo neste estudo.

Com base nos estudos funcionais *in vitro,* estas novas variantes apresentam um baixo potencial oncogénico, resultados estes consistentes com o fenótipo pouco agressivo observado em ambas as pacientes deste estudo.

Apesar da variante RET T636M não envolver diretamente um resíduo de cisteína, poderá induzir a uma alteração conformacional na proteína que poderá afetar uma ligação intramolecular localizada na vizinhança deste codão. Isto poderá ter um impacto na estrutura da proteína RET, bem como na sua ativação. Curiosamente, esta

variante está localizada na vizinhança de certos codões que, quando mutados, estão associados a fenótipos mais agressivos.

Por outro lado, a variante RET C515W envolve diretamente um resíduo de cisteína, podendo a sua perda ter consequências diretas na ativação do recetor RET.

Indivíduos com mutações pouco patogénicas no gene RET podem necessitar de uma segunda mutação germinal ou somática para desenvolver um fenótipo mais agressivo, que leve à expressão clínica da doença. Esta hipótese foi levantada para a paciente B que possuía a variante em homozigotia, mas foi descartada quando se descobriu que uma prima, que possuía a mesma variante embora em heterozigotia, veio igualmente a desenvolver CMT, sugerindo que apenas um alelo mutado é suficiente para a manifestação da doença.

Fatores externos como o estilo de vida de cada indivíduo ou do próprio ambiente em que este se encontra inserido, adicionado ao desenvolvimento tardio da doença, bem como à sua baixa penetrância, são fatores que provavelmente contribuem para as variações na expressão clínica da doença, sendo assim difícil de perceber o efeito da mutação só por si.

Apesar de serem necessários estudos funcionais adicionais, a caracterização destas duas novas variantes contribui, não só, para o conhecimento dos mecanismos moleculares inerentes ao desenvolvimento de CMT nestas famílias, como também para a optimização do diagnóstico e o aconselhamento clinico de CMT hereditário.

Palavras-chave: RET, CMT, tiróide, carcinoma e mutação

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List of nomenclatures, abbreviations and acronyms

ANOVA	Analysis of Variance
Arg	Amino acid arginine
ARTN	Artemin
ATC	Carcinoma anaplásico da tiróide
C	Amino acid cysteine
cDNA	Complementary DNA
CMT	Carcinoma Medular da tiróide
CMTF	Carcinoma medular da tiróide familiar
CRD	Cysteine-rich domain
Cys	Amino acid cysteine
DMEM	Dulbecco's modified essential medium
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide trihosphates
DT	Doubling times
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ENS	Enteric nervous system
FACS	Fluorescence-activated cell sorting
FMTC	Familial Medullary Thyroid Carcinoma
FNMTC	Familial Nonmedullary Thyroid Carcinoma
FTA	Follicular Thyroid Adenoma
FTC	Follicular Thyroid Carcinoma
GDNF	Glial cell line-derived neurotrophic factor
GFL	GDNF-family ligands
GFRα	GDNF-family receptor-alpha
GPI	Glycosylphosphatidyl inositol
HSCR	Hirschsprung's disease
KDa	Kilodalton

M Amino acid methionine

MEN Multiple Endocrine Neoplasia

Met Amino acid methionine

MTC Medullary Thyroid Carcinoma

NCBS Newborn calf serum

NMTC Non-medullary Thyroid Carcinoma

NRTN Neurturin

PAGE Polyacrylamide gel electrophoresis

PBS Phosphate Buffered Saline

PCR Polimerase Chain Reaction

PDTC Poorly Differentiated Thyroid Carcinoma

PI Propidium iodide

PSPN Persephin

PTC Papillary Thyroid Carcinoma

R Amino acid arginine

RAS Rat sarcoma virus homolog

RET Rearranged during transfection

RNA Ribonucleic acid

RNAse Ribonuclease

RT Room Temperature

RTK Receptor tyrosine kinase

RT-PCR Reverse Transcription-Polimerase Chain Reaction

S Amino acid serine

SDS Sodium dodecyl sulfate

Ser Amino acid serine

T Amino acid threonine

Taq Thermus aquaticus

TBE Tris-borate-EDTA

TBST Tris Buffered Saline with Triton

Thr Amino acid threonine

TK Tyrosine kinase

TKD Tyrosine kinase domain

Trp Amino acid tryptophan

UV Ultraviolet

W Amino acid tryptophan

WDTC Well Differentiated Thyroid Carcinoma

WT Wild type

Introduction

1. Tumorigenesis

After years of great scientific advances, research in cancer area became rich and complex, showing that this disease involves dynamic changes in the genome. Increasing evidence show that tumorigenesis in humans is a process involving several interconnected events associated with genetic alterations, reflecting a progressive transformation of normal cells into highly malignant ones. This transformation compromises cells' biological functions, such as proliferation, differentiation and cellular death.

Hanahan e Weinberg proposed that cancer cells acquire six biological capabilities during the multistep development of human tumors. These six traits are shared by nearly all cancers and are considered to represent the hallmarks that govern the conversion of normal cells into cancer cells (Hanahan and Weinberg, 2000, 2011). Those include growth signal autonomy, insensitivity to anti-growth signals, apoptosis evasion, replicative immortality, angiogenesis induction and tissue invasion and metastasis (Hanahan and Weinberg, 2000, 2011). The acquisition of these hallmarks is associated with genome instability, which leads to increased mutability, and to inflammation, which promotes multiple hallmark functions. Moreover, the capabilities of evading immune destruction and of reprogram the energy metabolism, by using abnormal metabolic pathways to generate energy, have been recently added to the cancer's hallmarks list. Furthermore, it is believed that the tumor microenvironment (normal cells, molecules, and blood vessels that surround and feed a tumor cell) also contributes significantly to the acquisition of the hallmark traits.

Cancer is a leading cause of disease worldwide. Overall, according to estimates from the International Agency for Research on Cancer, 12.7 million new cancer cases and 7.6 million cancer deaths were reported in 2008. The most commonly diagnosed cancers worldwide are lung (1.61 million, 12.7% of the total), breast (1.38 million, 10.9% of the total) and colorectal cancers (1.23 million, 9.7% of the total). The most common causes of cancer deaths are lung cancer (1.38 million, 18.2% of the total),

stomach cancer (738,000 deaths, 9.7%) and liver cancer (696,000 deaths, 9.2%) (Ferlay et al., 2010). Thyroid cancer is a rare type of cancer corresponding to approximately 1.0%–1.5% of all new cancers diagnosed each year in the USA. It is, however, the most common endocrine cancer. Currently it is the fifth most common cancer in women. It is estimated that at 2019, papillary thyroid cancer will become the third most common cancer in women of all ages after breast and lung cancer and will become the second most common cancer in women under age 45, after breast cancer (Aschebrook-Kilfoy et al., 2013). Moreover, its incidence has continuously increased in the last three decades all over the world. (Di Cristofano, 2013)

2. Thyroid gland

Thyroid gland is part of the endocrine system, is located along the front of the trachea below the thyroid cartilage also known as the Adam's apple. It is composed by two lobes connected by an isthmus (Figure I.1). The adult gland varies in size and appearance according to functional activity, gender, hormonal status, and iodine intake (Muro-Cacho and Ku, 2000).

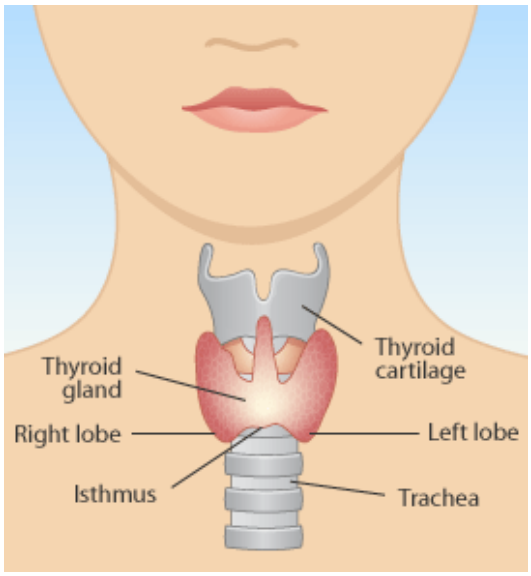


Figure 1 - Thyroid gland and its anatomical position. (www.abc.net.au/health/library/stories/2005/06/16/1831822.htm)

The thyroid gland is a very important organ for the development of vertebrates as it synthesizes hormones, such as tetraiodothyronine (thyroxine or T4) and triodothyronine (T3), that are essential for growth, development and survival (Nitsch et al., 2010).

In higher vertebrates the thyroid develops from the anterior foregut endoderm in which progenitor cells expressing critical transcription factors, assemble to form the thyroid bud. The growing bud subsequently delaminates from the pharyngeal endoderm and moves downward to the final anatomical position of the thyroid.

Thereafter, the solid mass separates and forms the two lobes, which are located on both sides of the trachea (Fagman and Nilsson, 2010a, 2010b).

Thyroid gland is mostly composed mostly by follicular cells and parafollicular cells (also called C cells). Follicular cells are the most abundant and are responsible for the formation of spherical structures, the follicles, where iodine is stored; C cells surround follicular cells and are responsible for calcitonin production (Ljungberg et al., 1983; Muro-Cacho and Ku, 2000).

3. Thyroid neoplasia

Endocrine neoplasias comprise a wide variety of tumor types. Thyroid carcinoma is the most common endocrine cancer, being more common in females than in males. Its incidence has continuously increased in the last three decades all over the world. Currently, constitute about 1 % of all carcinomas and, annually, 122,000 new cases are estimated to occur worldwide (Brown et al., 2011; DeLellis, 2004; Maenhaut et al., 2008). Some possible explanations for this significant increase are the improvement of diagnostic techniques used in the detection of thyroid cancer, improvement of medical imaging techniques or even the effect of radiation (Kent et al., 2007; Peterson et al., 2012).

There are, however, some factors that may influence the development of this type of carcinomas, such as deficiency in levels of iodine, hormonal or genetic factors, or even the individual lifestyle (diet, physical exercise, etc.) (Kent et al., 2007; Kondo et al., 2006; Peterson et al., 2012)

Thyroid neoplasia can be benign, called adenomas (e.g. Follicular Thyroid Adenoma - FTA) or malignant, called carcinomas (Muro-Cacho and Ku, 2000).

Thyroid carcinomas are classified according to the cell type from which they develop: carcinomas derived from thyroid C cells are called medullary thyroid carcinomas (MTCs); carcinomas derived from follicular cells are called non-medullary thyroid carcinomas (NMTCs) (Muro-Cacho and Ku, 2000).

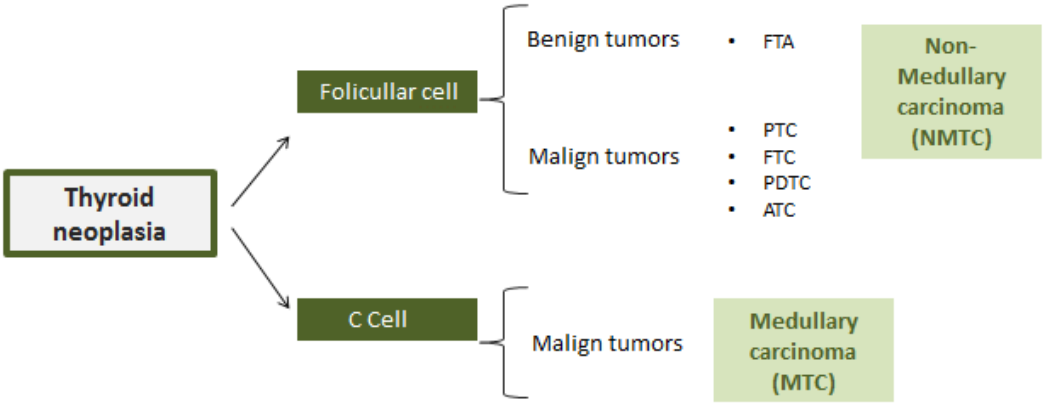


Figure 2 - Classification of thyroid neoplasia (adapted from Muro-Cacho and Ku, 2000a)

3.1. Non-Medullary Thyroid carcinoma

NMTCs represent the most prevalent form of thyroid cancer, accounting for approximately 95% of all cases. Of these, approximately 5% are thought to be of familial origin (familial non-medullary thyroid cancer - FNMTC) (Khan et al., 2010). NMTCs are classified according to their histopathological characteristics and degree of differentiation.

The well differentiated forms of thyroid carcinomas (WDTC) include the papillary thyroid carcinoma (PTC, accounting for 85% of cases) and the follicular thyroid carcinoma (FTC, accounting for 11% of cases). The anaplastic thyroid carcinoma (ATC) is the most aggressive form of thyroid cancer with a very poor prognosis accounting for 1% of cases. ATC can occur *de novo* or may derive from dedifferentiation of a formerly existent PTC or FTC (Taccaliti et al., 2012). In fact, there is also an intermediate stage between the well-differentiated (papillary and follicular) and the totally undifferentiated (anaplastic) called poorly differentiated thyroid carcinoma (PDTC) (Muro-Cacho and Ku, 2000; Sherman, 2003). The PDTC form, itself, together with the presence of differentiated areas in carcinomas with undifferentiated zones (DeLellis, 2004) makes PDTCs to be regarded as an intermediate stage in the progression from WDTC to ATC (Kondo et al., 2006). This progression model suggests that cellular dedifferentiation results in the sequential accumulation of events, such as gene mutations, chromosomal abnormalities and epigenetic alterations. It is common to

find mutations in certain genes that are not present in WDTC such as CTNNB1 (Beta-catenin), TP53 (tumor protein P53) and PI3KCA (phosphoinositide-3-kinase, catalytic, alpha polypeptide) (DeLellis, 2004; Sastre-Perona and Santisteban, 2012; Soares et al., 2011), suggesting that these changes occur during cellular dedifferentiation (Soares et al., 2011). Thus, exclusive mutations in more aggressive forms of thyroid carcinoma could represent additional genetic events associated with a more advanced stage in tumoral progression; conversely, common mutations in WDTC, PDTC and ATC are likely to be associated with early events associated with tumor initiation (Kondo et al., 2006).

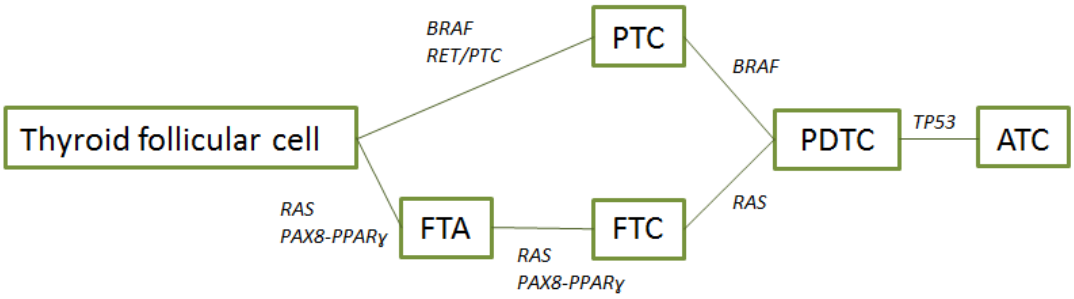


Figure 3 - Schematic representation of the progression of thyroid carcinomas (Adapted from Kondo et al., 2006)

3.2. Medullary Thyroid Carcinoma

MTC arises from calcitonin-producing neural crest-derived parafollicular (C) cells of the thyroid and accounts for 5–8% of all thyroid cancers (Cerrato et al., 2009).

This type of cancer usually appears in adults between 30 and 60 years, with a slight predominance in women (Kondo et al., 2006). The vast majority of these tumors is sporadic (about 75% of cases), but about 25% correspond to hereditary (autosomal dominant) forms (DeLellis, 2004; Moley and DeBenedetti, 1999). In this case, it can occur in an isolated form, in which MTC is the only component present in a family (*Family Medullary Thyroid Carcinoma* – FMTC) or in the context of multiple endocrine neoplasia syndrome (MEN), subdivided in MEN2A and MEN2B (Dvorakova et al., 2008; Zbuk and Eng, 2007).

FMTC is considered by some to be a variant of MEN2A while for others this is viewed as a distinct syndrome (Figlioli et al., 2013).

Germline mutations of the RET proto oncogene (Rearranged During Transfection) are present in about 98% of the family syndromes associated with MTC (98% of cases of MEN2A, 95% of MEN2B and 88% of FMTC) (Arighi et al., 2005). Somatic mutations in RET were also found in sporadic MTC forms (Marsh et al., 1996).

Although RET mutations are crucial to the development of MTC, there are, however, cases of MTC that are negative for mutations in this gene. In fact, mutations in the proto-oncogene RAS (rat sarcoma virus homolog) were found in 68% of RET-negative sporadic MTC. This is also indicative that mutations in proto-oncogenes RET and RAS represent alternative genetic events in tumorigenesis of MTC (Moura et al., 2011).

4. The proto-oncogene RET

RET oncogene was identified by Takahashi et al., who reported a novel gene re-arrangement with transforming activity in NIH 3T3 cells transfected with human lymphoma DNA (Arighi et al., 2005; Takahashi et al., 1985).

This proto oncogene encodes a membrane receptor with tyrosine kinase activity. Receptor tyrosine kinase (RTK) constitute a large family of receptors that, in response to their ligand activation, are potent mediators of cell motility, proliferation, differentiation, and survival (Phay and Shah, 2010; Zhou et al., 2012). The receptor is mainly expressed in neural crest-derived tissues, such as C-cells of the thyroid, adrenal medullary cells, colonic ganglia cells and parathyroid cells. It is required for maturation of several cell lineages of the peripheral nervous system, kidney morphogenesis and spermatogenesis (Arighi et al., 2005; Zhou et al., 2012).

RET is located on chromosome 10q11.21 and has 21 exons. Gene homologous were found in upper and lower vertebrates and also in *Drosophila melanogaster* (Hahn and Bishop, 2001).

The natural splicing of the RET gene codes for multiple proteins, including 3 major isoforms that have 9 (RET9), 51 (RET51) or 43 (RET43) distinct amino acids at their C-

termini. RET 9 and RET 51 are the most conserved among species and considered to be the most relevant (Carter et al., 2001; Salehian and Samoa, 2013).

Loss-of-function mutations of human *RET* have been implicated in Hirschsprung's disease (HSCR), a rare and congenital anomaly of the enteric nervous system (ENS) that occurs with an average incidence of 1 into 5000 live births (Rusmini et al., 2013).

In contrast, mutations in RET gene that lead to constitutive activation of RET tyrosine kinase activity are responsible for the development of MTC and for the inherited cancer syndrome, MEN2.

4.1. Activation of RET receptor tyrosine kinase

RET protein is composed of three domains: an extracellular ligand-binding domain (that contains four cadherin-like repeats as well as a highly conserved cysteine-rich region), a hydrophobic transmembrane domain, and a cytoplasmic region containing the TK domain (Arighi et al., 2005; Hahn and Bishop, 2001; Phay and Shah, 2010; Sherman, 2003; Zhou et al., 2012).

A tripartite cell-surface complex is necessary for RET signaling. RET is the receptor of a multimolecular complex that binds the growth factors of the glial cell line-derived neurotrophic factor (GDNF) family. GDNF-family ligands (GFLs) bind to and activate RET when bound to GDNF-family receptor-alpha (GFRα) proteins. GFRαs are ligand-binding co-receptors with no intracellular or transmembrane domains, and are anchored to the cell surface by glycosylphosphatidyl inositol (GPI)-linkage.

The primary ligands for the co-receptors GFRα1, GFRα2, GFRα3 and GFRα4 are GDNF, NRTN, ARTN and PSPN, respectively (Arighi et al., 2005; Wells and Santoro, 2009).

The GFL and GFRα association leads to RET dimerization to form a GFL(X2)-GFRa(X2)-RET(X2) heterohexamer complex. RET receptor dimerization induces the trans-autophosphorylation of specific tyrosine residues within the TK domain. This ultimately leads to the phosphorylation of target-proteins, which are able to activate multiple signaling pathways (Arighi et al., 2005; Phay and Shah, 2010).These pathways are mediators of cell motility, proliferation, differentiation, and survival. These include: RAS/RAF/ERK1/2 (*rat sarcoma oncogene/rapidly accelerated fibrosarcoma/extracelular*

regulated kinase 1/2), PI3K/AKT (*the phosphatidylinositol 3-kinase/protein kinase B*), JNK (*c-Jun N-terminal kinase*), p38 (Mitogen-Activated Protein Kinase 14), ERK5 (*enigma extracellular regulated kinase 5*), CREB *(cAMP-responsive element-binding protein)* and STAT3 (*signal transducer and activator of transcription 3*) pathways (Arighi et al., 2005; Gujral et al., 2008; Prazeres et al., 2011a).

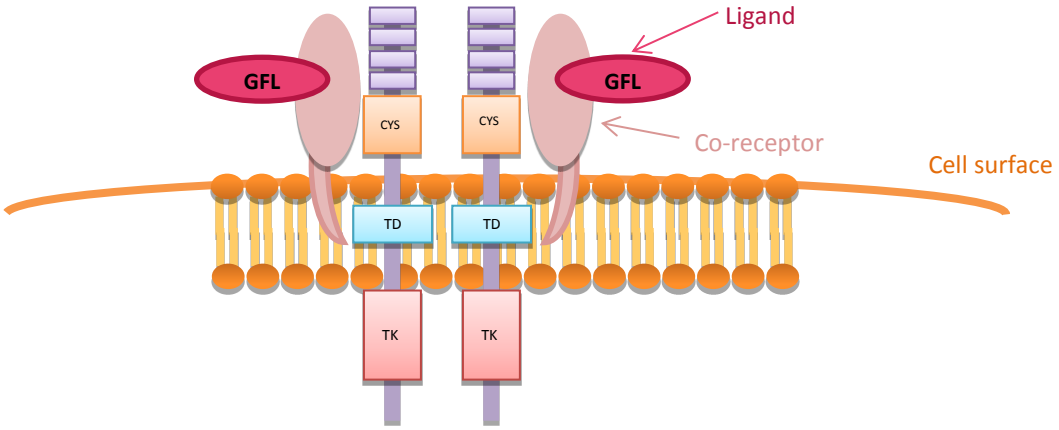


Figure 4 - Schematic representation of Ret protein

4.2. RET mutations

Genetic changes that occur in more than 1% of the population are called polymorphisms. Although they are common enough to be considered a normal variation in the DNA, some of these variations may influence the risk of developing certain disorders. On the other hand, genetic changes occurring in the population with a frequency of < 1% are designated as mutations and these, due to their low frequency, have an increased potential of being pathogenic (Kochański, 2007).

Gene mutations may occur in the DNA of individual cells at some time during a person's lifetime (somatic mutations) or can be inherited from a parent (germline mutations), being present throughout a person's life in virtually every cell in the body.

Germline point mutations of RET are responsible for FMTC and for all autosomal dominant MEN2 cancer syndromes, MEN2A and MEN2B. These mutations are observed in 88% of FMTC and in 98% of MEN2A and 95% of MEN 2B (Arighi et al., 2005; Figlioli et al., 2013).

Numerous mutations in RET have been described associated with different levels of pathogenicity. Most of the RET mutations described correspond to heterozygous *missense* sequence changes (point mutations that change a single nucleotide resulting in a codon that codes for a different amino acid) in exons 5, 8, 10, 11, and 13-16.

Germline mutations are almost exclusively distributed within only two domains of RET: the extra-cellular cysteine-rich domain (CRD) and the intracellular tyrosine kinase domain 1 (TKD1). They mainly affect codons 609, 611, 618, 620, and 634 (all encoding for cysteine in the CRD), and codons 768 (exon 13) and 804 (exon 14) of the TKD1 (Figlioli et al., 2013; Zhou et al., 2012).

In MEN 2A, mutations at codon 634 account for 85% of all the mutations identified so far, being the cysteine to arginine (C634R) substitution the most common amino acid change (50%). Mutations at codons 609, 611, 618, and 620 (within exon 10) account for a further of 10 to15%.

In MEN 2B, about 95% of patients carries the substitution at codon 918 (M918T) within exon 16.

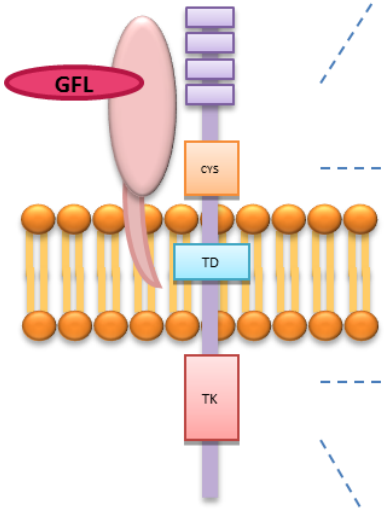
The mutation spectrum for FMTC is less defined. Since 1993, a total of 39 different RET germline mutations were identified in FMTC patients from different families (Figlioli et al., 2013; Zhou et al., 2012).

5. RET genetic screening

Genetic testing can identify patients affected by MEN2A and 2B and familial MTC, allowing early diagnosis and possible cure (Giuffrida and Gharib, 1998).

RET genetic analysis is a safe and accurate measure of hereditary MTC diagnosis. Before RET genetic analysis was available, the first-degree relatives of hereditary MTC patients who have a 50% chance of inheriting the predisposing gene had to undergo repeated biochemical screenings in order to prevent the disorder. Since biochemical screening results were ambiguous, the disorder was often missed or misdiagnosed, or diagnosed at an advanced stage. The implementation of RET genetic screening provides a reliable methodology that enables the identification of at-risk individuals. This allows more effective and timely clinical screenings for disease to be diagnosed at early stages and early surgical intervention (Figlioli et al., 2013).

Molecular genetic testing of RET identifies disease-causing mutations in up to 98% of individuals with MEN2 or FMTC. There are, however, RET germline mutations detected in index cases routinely submitted to RET genetic analysis, for which the level of pathogenicity is unknown. Evaluating the biological consequences of such mutations in disease development is extremelly relevant for defining mutation's codon-specific risk level. This may have important implications for familial genetic screening and carriers follow-up.



Mutated codon	Exon	Clinical syndrome
533	8	FMTC
606	10	FMTC
609		FMTC/MEN 2A
611		FMTC/MEN 2A
618		FMTC/MEN 2A
620		FMTC/MEN 2A
630	11	FMTC
632/633/634		MEN 2A
634		MEN 2A
640		MEN 2A
641		MEN 2A
648		MEN 2A
768	13	FMTC
781		FMTC
790/791		FMTC
804	14	FMTC
844		FMTC
883	15	FMTC/MEN 2B
891		FMTC
904		FMTC/MEN 2B
912	16	MEN 2B
918		MEN 2B
922		MEN 2B

Figure 5 - Genotype / phenotype correlation of different mutations of Ret (Adapted from Arighi et al., 2005; Figlioli et al., 2013; Phay and Shah, 2010; Zbuk and Eng, 2007)

6. Objectives

The main objective in this work is to evaluate the oncogenic potential of two new RET germinal variants detected in two MTC patients from our institution. These new variants have not been described so far in literature.

A set of functional *in vitro* approaches, such as focus formation, cell growth, cell cycle and cell migration assays were designed to test the oncogenic potential of these two variants, in order to clarify their role in disease etiology and development.

Materials and methods

1. Patients

Two novel germinal RET variants were identified in two MTC patients from our institution (hereafter referred as A and B), following RET genetic screening, after informed consent. The newly identified variants have not yet been described in the literature. Patient A was a 79 year-old woman without a familial history of FMTC/MEN2A/MEN2B who underwent thyroidectomy for unifocal MTC. RET genetic testing identified a heterozygous variant at codon 636 (ACG/ATG) in exon 11 that results in a threonine-for-methionine substitution in the extracellular region of the RET protein. Only another family member was tested but was found to be negative for the new variant: the proband's 50 year-old daughter who presented multinodular goiter.

Patient B was a 60 year-old woman who underwent total thyroidectomy for multifocal MTC. Genetic testing identified, in peripheral blood DNA, a homozygous variant in exon 8, at codon 515 (TGC/TGG), that causes a substitution of a cysteine for a tryptophan residue in the extracellular domain of the RET protein. Her parents were first-degree cousins. This variant was also found, yet in heterozygosity, in eight of the ten proband's relatives hither to study. None had any symptoms suggestive of hereditary MTC or MEN 2 except for one, a cousin who also developed MTC at age 63.

2. *In silico* analysis

Functional analysis *in silico* was used to predict the impact of the newly identified RET variants on the structure and function of the encoded proteins so as to infer its pathogenic potential. The bioinformatic analysis was performed by using the following software tools:

Polyphen2 (http://genetics.bwh.harvard.edu/pph2)

SIFT/PROVEAN (http://sift.jcvi.org/www/SIFT_enst_submit.html)

Mutation Taster (http://www.mutationtaster.org)

3. Plasmids and construction of expression vectors

The pcDNA3.1/Zeo(+) plasmids, carrying the human wild-type RET cDNA sequence (RET WT – encoding the full-length RET long isoform of 1114 amino acids containing a myc- epitope-tag at its C-terminus), or the corresponding RET variant containing the MEN2A-causing mutation Cys634Arg (RET C634R), were kindly donated by Dr. Masahide Takahashi and have been described previously (Fukuda et al., 2002).

Constructs carrying the RET new variants identified in our lab were generated by site directed mutagenesis of RET-WT construct. RET C515T construction (RET missense alterations TGC→TGG at codon 515 - Cys515Trp) had been already accomplished by Dr Branca Cavaco's lab before the beginning of this master's course and was generously provided due course of this work. To generate RET T636M construct, an in vitro oligonucleotide mutagenesis system (QuikChange site-directed mutagenesis; Stratagene) was used following manufacturer's instructions to introduce the RET missense alteration ACG→ATG at codon 636 (Thr636Met), using the mutagenic primers foward (5´gagctgtgccgcatggtgatcgcagcc) and reverse (5'gctgcgatcaccatgcggcacagctc).

Plasmid DNA preparations were obtained using the MAXI PREP Kit (Qiagen) and concentration was determined spectrophotometrically in Nanodrop 2000 (Thermo Scientific). Coding sequences of RET variants were confirmed by direct sequencing.

4. Reverse transcription - Polymerase Chain Reaction and Sequencing

The expression of transfected RET constructs in NIH 3T3 cells, was monitored by reverse transcription-polymerase chain reaction (RT-PCR).

Total RNA was obtained from cell lysates using RNeasy Mini Extraction kit (74104, Qiagen), according to manufacturer's protocol and 2 µg were reverse transcribed using random primers and SuperScript II (Invitrogen^{TM}) (Appendix, Supplementary Table 1, 2 and 3).

A RET cDNA fragment, encompassing exons 8 to 11, was then amplified by PCR with specific primers (Primer foward: 5'GGGGATCCTGTTTGTGAATG; Primer reverse: 5'TTGTGGGCAAACTTGTGGTA) (Appendix, Supplementary Table 4 and 5).

PCR products were resolved by electrophoresis on a 2% (w/v) agarose gel in 1X Tris-borate-EDTA (TBE) buffer (diluted from 10X TBE, EC-860, National diagnostics) and stained with 0.05% (v/v) ethidium bromide.

The separated DNA was visualized under UV light and image acquired in a ChemiDoc XRS System (BIO-RAD).

Direct sequencing of RT-PCR products was performed in order to confirm the integrity of RET construct-derived expression. Sequencing was performed with BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and data analyzed in ABI Prism 3130 Genetic Analyzer.

5. Cell culture

In this study we used the commercially available mouse fibroblast NIH 3T3 cell line. Cells were maintained at 37°C in a humidified 5% CO_2 environment in an appropriate chamber (NUAIRE™ US AUTOFLOW CO_2 water-jacketed incubator) in complete DMEM medium - Dulbecco's modified essential medium (DMEM) (GIBCO™) supplemented with 10% (v/v) newborn calf serum (NCBS) (GIBCO™). Cells were subcultured at least twice per week at 80% confluence: cells were detached by incubation with 1X 0.05% trypsin-EDTA (Invitrogen™) at RT for 5 minutes and 1:3 of cell suspension was added to new culture vessel. Whenever necessary, cell concentration was determined using a hemocytometer (Bürker counting chamber).

6. NIH 3T3 Transfections

Transfection assay is the process of introducing nucleic acids into eukaryotic cells by nonviral methods.

In transient transfections, foreign DNA does not integrate into the cell genome and transfected genes are expressed for a limited time. In stable transfections, however, cells have integrated foreign DNA into their genome and, consequently, descendants of these transfected cells will also express the transfected gene (Kim and Eberwine, 2010). Stable transfected cells can be selected by taking advantage on the co-integration of an antibiotic-resistance gene (usually present as an independent

transcriptional unit on the transfected expression vector) and the use of the corresponding selective antibiotic. For stable transfections, plasmids were linearized with the restriction enzyme PVU I (Invitrogen™), which recognizes a single restriction site on plasmid backbone. The use of linearized vectors in stable transfections will increase the odds of vectors integrate into the genome without disrupting the gene of interest or other elements required for protein expression.

Transfections were performed using the cationic lipid-based transfection reagent Lipofectamine™ 2000 (Invitrogen™). Briefly, $5x10^5$ NIH 3T3 cells were seeded, 24h before transfection, in 35-mm dishes (Nunclon). Just before transfection, cells were fed with new fresh complete DMEM medium. Plasmid DNA (2 µg) was diluted into 125 µl OPTI-MEM (GIBCO™); in an additional tube, 4 µl of transfection reagent was also diluted in 125 µl of OPTI-MEM and incubated 5 minutes at room temperature (RT). Diluted DNA and lipofectamine were then mixed gently and incubated for 20 minutes at RT to allow the formation of DNA-lipofectamine complexes. After incubation, 250 µl of transfection mixture was added to each culture dish.

In order to select stable transfectants, Zeocin™ (Life Technologies) was used as selection antibiotic at the concentration of 500 µg/mL. Resistance to Zeocin™ is conferred by expression of the Sh ble gene present in the RET expression vector pcDNA3.1/Zeo(+).

7. Western Blotting

Western Blotting is a conventional technique used to detect a specific protein present in a protein extract. Proteins present in the extract are usually separated by length using denaturing sodium dodecyl sulfate-poliacrilamyde gel electrophoresis (SDS-PAGE). Proteins are then transferred to a solid support (typically a nitrocellulose or a polyvinylidene fluoride - PVDF membrane), where they can be detected using protein specific antibodies (primary antibodies) associated with an enzyme-conjugated secondary antibody that recognizes the primary antibody. Secondary antibodies are usually conjugated with horseradish peroxidise that is able to catalyse a chemiluminescent reaction that produces a signal allowing the visualization of target protein location in the membrane.

This technique enables the quantification of relative amounts of a particular protein present in different samples. We used this technique to monitor and quantify the exogenous expression of RET in NIH 3T3 transfected cell pools. Transfected RET protein variants were detected using an antibody specific for the *c-myc* epitope tag present in RET C-terminus. Detection of human β-actin (43 kDa) was used as loading control.

To obtain protein extracts, cells were transferred to a 2ml eppendorf tube and centrifuged at 1500 rpm for 5 minutes. Then, they were washed with PBS (*Phosphate Buffared Saline* -Mg2+/Ca2+; GIBCO^{TM}, *Invitrogen Corporation*, UK) and centrifuged at 1500 rpm for 5 minutes. Lysis buffer was prepared (Appendix, Supplementary Table 6), and added to cells' pellet (usually 1x10^6 cells are efficiently lysed in 100µl). The lysates were incubated at 100ºC for 5 minutes for protein denaturation, and then stored at -20ºC.

Equivalent amounts of total protein extract were separated in a 10% SDS-PAGE (20 mA, 2h, Appendix, Supplementary Table 6). A molecular weight marker (SDS-PAGE Standards - BIO-RAD) was used, designed to estimate the expected location of the protein band of interest. The samples were transferred to a PVDF membrane (*Polyvinylidene fluoride; BIO-RAD)* using a Mini Trans-Blot® Electrophoretic Transfer Cell (*BIO-RAD*) apparatus at 0.22A for 2h, in Blott buffer (Appendix, Supplementary Table 6).

Proteins transferred to PVDF membrane were then stained with Coomassie Brilliant Blue, to monitor the effectiveness of the transfer. A 30 min incubation in Destain solution (Appendix, Supplementary Table 6) was used to remove excess dye and membrane was then rinsed 3 times in TBST (Appendix, Supplementary Table 6) with stirring.

In order to prevent non-specific protein-antibody binding membranes were incubated for 1h in 4 % (w/v) of powder non-fat milk diluted in TBST.

Membranes were then probed with mouse monoclonal anti-β-actin (clone, Sigma) at 1:3000 dilution and mouse monoclonal anti-myc tag (clone 9E10, Sigma) at 1:500 dilution. For detection we used a secondary peroxidase-conjugated anti-mouse IgG (Thermo) at 1:3000. Incubations with antibodies were carried out in TBST-milk (Appendix, Supplementary Table 6) for 1h30min at RT. Immunoreactive bands were

detected by chemiluminescence using ECL Western blotting substrate (Appendix, Supplementary Table 6), followed by exposure and image acquisition in a ChemiDoc XRS System (*BIO-RAD*). Images were analysed using *ImageJ* software.

8. Focus formation assay

Focus formation assays have been used extensively to evaluate the transforming potential of a wide variety of genes. NIH 3T3 mouse fibroblasts have been well-established as a biological model to measure the ability of an exogenously expressed gene to promote cell growth transformation. Normal cells in culture are inhibited by contact, blocking their division when a critical density is reached and forming a confluent monolayer; transformed cells, however, are not affected by this phenomenon and lose their contact inhibition. This leads to the formation of three-dimensional foci, which sometimes can be visualized macroscopically. The number and dimension of the foci formed could be a measure of the oncogenic potential of the exogenously expressed gene.

For focus formation assay, NIH 3T3 cells were transfected with the RET constructs carrying the different RET variants (RET T636M, RET C515W, RET C634R and RET-WT) as well as with pcDNA3.1/Zeo(+)-empty vector and H-Ras V12- expressing construct.

Transfected pools were splited into two 100-mm dishes (Nunclon). Complete DMEM culture medium (supplemented with 1% antimycotic-antibiotic (GIBCO^{TM})) was changed every three days during approximately 4 weeks. After this period, the culture medium was replaced by DMEM with 5% NBCS, for 2 weeks, in order to pre-select transfected cells which were not affected by a decrease in NBCS levels. After these 6 weeks in culture, macroscopic foci could be visualized and were stained with crystal violet. Briefly, dishes were placed on ice and washed twice with cold PBS. Cells were fixed with ice-cold methanol for 30 min at -20ºC. Then, cells were stained with 0,5% crystal violet solution (0,5% crystal violet; 25% methanol) for 10 min at RT. Excess of dye was removed with destaining solution (10% methanol).

Formed foci were quantified by image analysis using *ImageJ* software. Both foci number and size were considered.

9. Cell cycle analysis by Flow Cytometry

Cell cycle analysis by flow cytometry is based on the ability of staining the cellular DNA in a stoichiometric manner (the amount of stain is directly proportional to the amount of DNA within the cell). Through this methodology, the nuclear DNA content of a cell is stained with a fluorescent dye and the stained material is measured at a high speed flow cytometer (FACS - Fluorescence-activated cell sorting). Since the cellular DNA content reflects cell cycling phases, this technique also enables the identification of cell distribution in a mixed proliferative population during the various phases of the cell cycle. Cell cycle comprises four distinct phases: the S phase where synthesis and duplication of nuclear DNA occur before division; the M phase where the process of cellular division itself (mitosis) occurs; the G1 and G2 phases that correspond to temporal gaps between mitosis and the onset of DNA synthesis (G1) and between the completion of DNA synthesis and the onset of mitosis (G2). Normal DNA content in G1 cells correspond to that of diploid cells; G2 and mitotic cells, on the other hand, have both twice the normal G1 DNA content and therefore cannot be discriminated based on these differences (Cecchini et al., 2012; Nunez, 2001); cells in the S phase have a DNA content just barely above their starting G1 content and this increases progressively until they complete the S phase with the G2 DNA content.

To perform cell cycle analysis by FACS, NIH 3T3 stably transfected pools were seeded in six-well tissue culture plates at a concentration of $3x10^5$/well in complete DMEM culture medium. After approximately 7 hours (when cells adhere) the medium was replaced by DMEM medium with 0,5% of NCBS and incubated at 37ºC and 5% CO_2 for 48h, for cell cycle synchronization. Subsequently, cells were maintained in complete DMEM culture medium and cell cycle was assessed 18h after serum-replenishment.

NIH 3T3 cells stably transfected with the several RET variants were harvested, fixed in 70% ethanol, incubated at 4ºC and then stained with a propidium iodide (PI) solution (Appendix, Supplementary Table 7). Firstly, cells were centrifuged at 1500 rpm for 5 minutes. Pellet was washed 3x in 1 mL of PBS, resuspended in 500 µL of PI solution and incubated at 37ºC for 2 hours. Following incubation, cell cycle acquisitions were performed through flow cytometry (FACScalibur - Becton Dickinson) and

analysed using FlowJo software. The fraction of cells in the various phases of cell cycle was assessed.

10. Cell growth assessment *in vitro*

Cell growth assays were performed to evaluate the impact of the exogenous expression of the several RET variants on NIH 3T3 cell growth rate.

NIH3T3 stably transfected pools were plated in twelve-well dishes (Nunclon) at a density of $4x10^3$cells/well in complete DMEM culture medium and let to adhere for approximately 8 hours. Cells were then subjected to serum starvation by replacing culture medium by DMEM supplemented with 0,5% NCBS and incubated at 37ºC, 5% CO_2, for 48h (to synchronize cell cycle). After this starvation period, cell number was determined (t=0h) and the culture medium was replaced by complete DMEM culture medium. Cells were counted every 24h for 5 days. A low initial cell concentration was used to assure that cell growth assessment could be performed at exponential phase of growth curve. Cell counting was performed using a Bürker counting chamber. Triplicates of each time point were performed and each cell dilution was assessed twice. Non-viable cells were excluded by using trypan blue staining. The population-doubling time was determined using Graph Pad Prism 4 software.

11. Cell migration - *In vitro* wound healing assay

In vitro wound healing assay is a commonly used model to study directional cell migration *in vitro.* This method mimics, to a certain extent, cell migration during wound healing process *in vivo* and is particularly suitable for studies on the effects of cell–matrix and cell–cell interactions on cell migration (Lee and Kay, 2006).

Pools of NIH-3T3 cells stably transfected with the different RET variants were seeded in twelve-well culture plates at a concentration of $2x10^5$ cells/well and maintained in complete DMEM culture medium. When cells reached 90 to 100% of optical confluence, they were treated with 5 µg/mL Mitomycin-C, (Sigma) a potent DNA cross-linker used to inhibit cell cycle, in serum-free-medium (Hsu et al., 2013; Leung et al., 2005). After the incubation period with Mitomycin-C, a tip of a micropipette (P200 tip) was used to wound the cell layer, creating linear scrapes. Then,

to remove floating cellular debris, the culture medium was replaced and cells were incubated for additional periods of time at 37ºC, 5% CO_2. Wound closure, corresponding to cell migration, was photographed when the scrape wound was introduced (t=0h) and at defined time points after wounding (t=8h, t=24h, t=32h).

12. Statistical analysis

Statistical analysis was carried out using GraphPad Prism 4 statistical software (San Diego, CA). When appropriate, values are expressed as mean ± SD. Statistical comparisons of proportions were made using the unpaired two-tailed Student's t-test. Cell growth curves were compared using two-way analysis of variance (ANOVA) followed by post-tests. Statistical significance was accepted at $P < 0,05$.

Results

1. RET functional analysis *In silico*

To predict the impact of RET missense point mutations T636M and C515W on the biological activity of the encoded proteins, *in silico* functional analysis was performed by using several software tools. Results are summarized in table III.1 While SIFT/PROVEAN predicted both variants to induce no phenotypic effect, Mutation taster considered them both to be possibly disease causing. Polyphen 2, on the other hand, predicted RET T636M to be benign and RET C515W to be possibly damaging.

Software	Mutation	Result
Polyphen 2	RET T636M	Benign/neutral
	RET C515W	Possibly damaging and disease causing
SIFT/PROVEAN	RET T636M	Benign/neutral
	RET C515W	Tolerable
Mutation taster	RET T636M	Disease causing
	RET C515W	Possibly damaging and disease causing

Table 1 –*In silico* prediction of the pathogenic potential of the two newly identified RET missense variants.

2. RET functional studies *in vitro*

To better address the transforming potential of the uncharacterized RET variants, RET T636M and RET C515W, *in vitro* functional studies were performed.

NIH 3T3 mouse fibroblast cell line has been widely used as a biological model to measure the ability of an exogenously expressed gene to promote cell transformation. Hence, NIH 3T3 cells were transfected with the different RET variants and RET expression was monitored by RT-PCR followed by direct sequencing. Protein expression levels were assessed by western blot.

Focus formation assays were carried out using non-established, non-selected, RET transfected cells, whereas cell cycle, cell growth and wound healing assays involved

the establishment of cells pools stably expressing the different RET variants. For these latter experiments, exogenous RET expression was compared between transfected pools and only pools with similar levels of RET transfected protein were used.

2.1. Focus formation assay

RET displays the classical features of a proto-oncogene. Several RET activating mutations have been shown to induce transformation of NIH3T3 fibroblasts, leading to the formation of foci. The ability of the newly identified RET variants, RET T636M and RET C515W, to transform NIH 3T3 fibroblasts was tested and compared to that of a strong RET oncogene associated with MEN 2A (RET C634R). The formation of three-dimensional foci in NIH 3T3 cells transfected with vectors expressing the different RET variants was assessed and normalized to that of RET wild-type (RET-WT). This assay was also performed with NIH 3T3 fibroblasts transfected with pcDNA3.1/Zeo(+) empty vector (Empty) and H-Ras V12 expressing construct, used as additional controls.

As shown in Figure III.1, and consistent to that described by others (Matos et al., 2008; Prazeres et al., 2011b) Ret C634R and Ras V12 showed the higher transforming activity (measured by number of foci formed), which is in agreement with their demonstrated oncogenic potential. The number of transformed foci induced by the new variants C515W and T636M was lower than that induced by C634R or Ras V12 being, however, significantly higher than that induced by RET-WT or Empty vector. This suggests that these new RET mutants hold, in fact, some transforming activity being able induce the loss of contact inhibition in non-transformed fibroblasts.

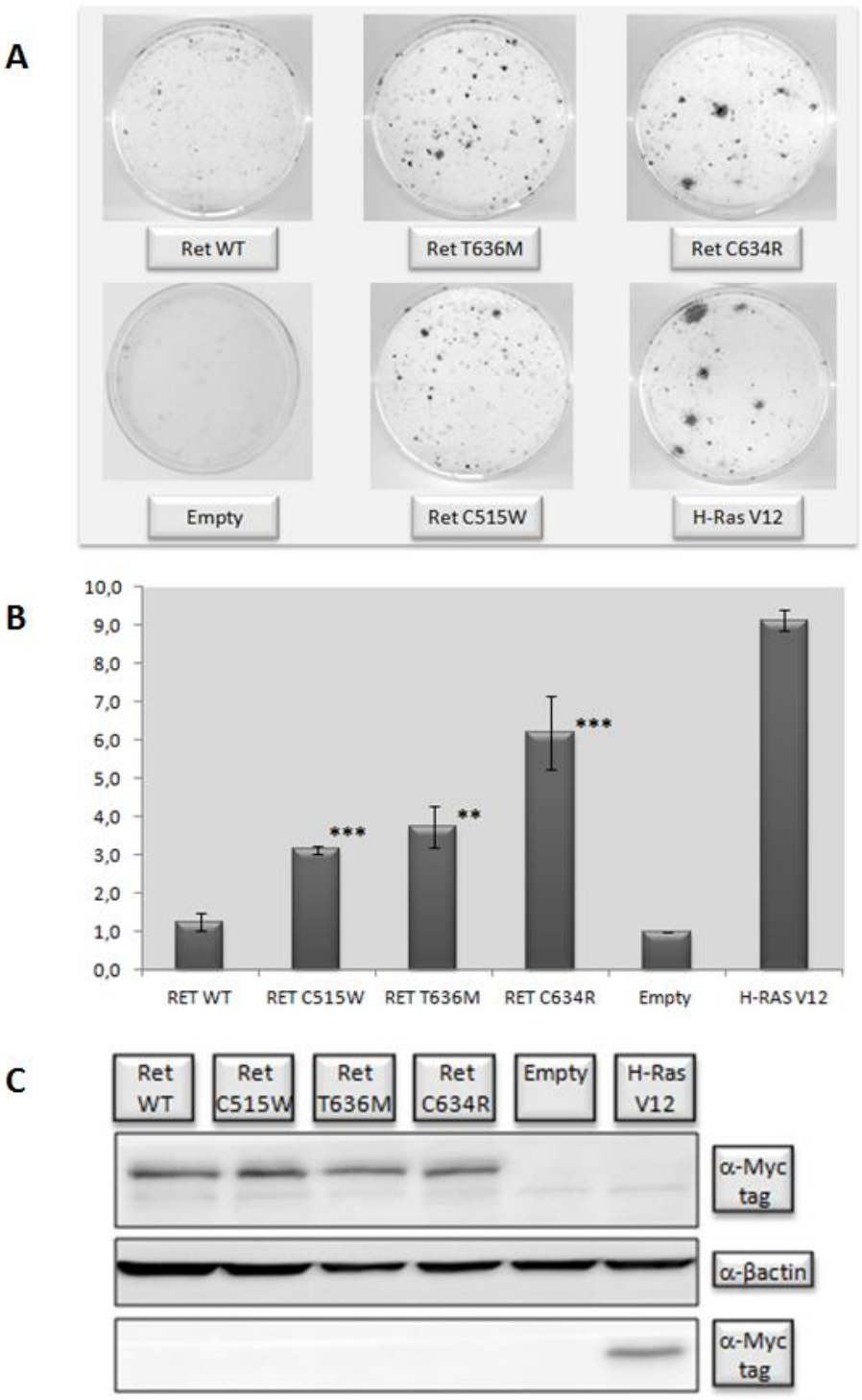


Figure 6 – Evaluation of the transforming capacity of RET variants in NIH 3T3 focus formation assay. Graphic showing the average number of foci generated in 3 independent assays, each performed in duplicate. Bars indicate standart deviation (A, B). Western blot representative of RET expression levels in transfected NIH3T3 pools(C). Statistically significant result, with *p<0.05, **p< 0.01, *** p<0.001

2.2. Cell cycle analysis

Cell cycle analysis by flow cytometry allows the determination of the distribution of a population of cells to the different stages of the cell cycle. Here we assessed whether the transforming activity of the RET new variants have an impact on cell cycle progression. NIH 3T3 selected pools, stably expressing the different RET variants, were used in this study. Cell cycle synchronization of transfected pools was done by serum starvation. After a 48h starvation period, cell cycle analysis of transfected pools has shown that cells were arrested at the G0/G1 phase, confirming cell synchronization (data not shown). Then, 18 h after cells were re-fed with 10% serum, cell cycle analysis was repeated. An increase in the transition of cells from G1/G0 to S phase was notorious for C634R pool (33,82%) when comparing to Empty (19,92%) and Ret-WT (17,69%) pools (Figure III.3). An intermediate increase of cells in the S phase was observed for Ret C515W (23,49%) and Ret T636M (28,58%) pools, comparing to negative (RET WT and Empty) and positive (RET C634R) controls. Also, an overall increase of cells in S and G2/M phases was observed for RET C515W (47,3%), RET T636M (45,4%), and RET C634R (49%) pools, comparing to negative controls (Empty - 37,2% and RET WT - 34,8%). This indicates that RET variants are likely to induce mitogenic activity of non-transformed fibroblasts, being able to stimulate cell proliferation *in vitro.*

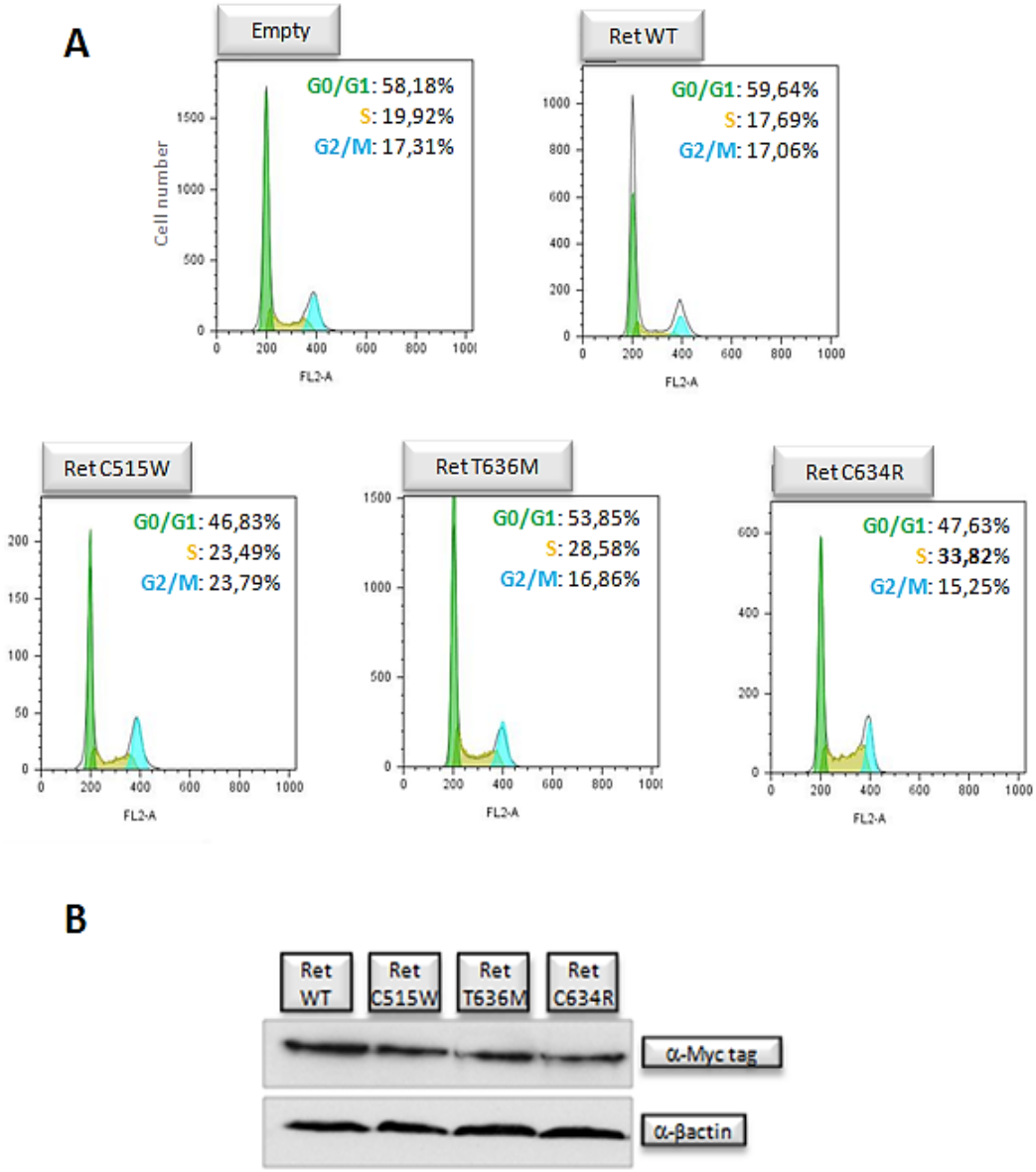


Figure 7 – Flow cytometry analysis showing the cell cycle distribution of NIH 3T3 cells (PI staining) (A). Western blot representative of RET expression levels in selected stable transfected NIH 3T3 pools (B).

2.3. Cell growth *in vitro*

To confirm whether the differences observed in cell cycle progression have an impact on cell growth and proliferation, cell growth curves were determined for NIH 3T3 stably transfected with the RET variants (Figure III.4).

After a 48h serum starvation period, cultures were serum-replenished and cells were counted every 24h for 5 days.

At day 3, we were already able to observe that cell number in C634R pool was significantly higher than in the other transfected pools (Figure III.4). At days 4 and 5, both C634R and RET new variants' pools, C515W and T636M, presented a significant increase in cell population comparing to RET WT pool. Still, RET C634R showed the highest increase. In addition, cell population doubling times calculated for each transfected pool are consistent with RET C634R pool having the highest cell growth rate (roughly 3 times the RET WT), followed by Ret C515W and Ret T636M pools (roughly twice the RET WT).

These data suggest that RET new variants are able to promote cellular proliferation, which is in accordance with results obtained from cell cycle assay.

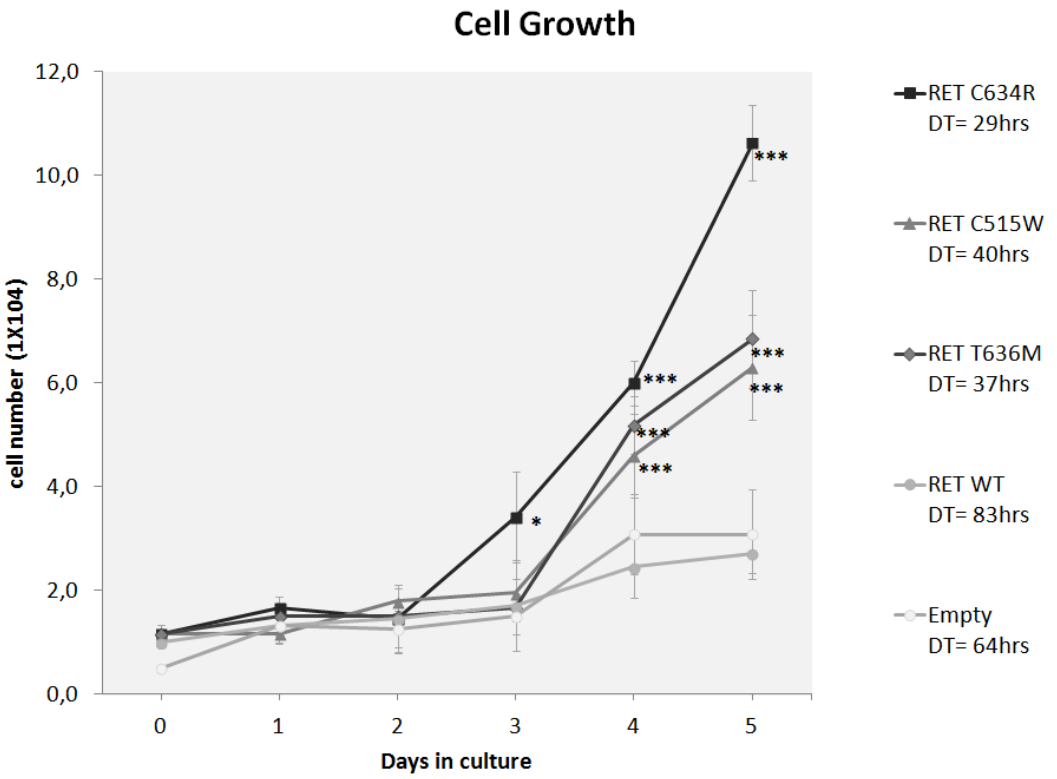


Figure 8 - Growth curve of RET variants in NIH 3T3. Graphic showing the cell number during 5 days in culture after 48 hours of serum starvation generated in 4 independent assays, each performed in triplicate. *Statistically significant result, with *p<0.05, **p< 0.01, *** p<0.001

2.4. Cell migration - *In vitro* wound healing assay

Cell migration, assessed by *in vitro* wound healing assay, is a common study for evaluate migration rate of cells.

This study was performed to investigate whether the RET new variants, Ret C515W and Ret T636M, were able to induce a migration phenotype in NIH 3T3 cells. Again, NIH 3T3 stably transfected with the RET variants were used and cell migration capacity was evaluated 8, 24 and 32 hours after wounding the cell layer (t=0). This assay was performed in mytomicin c- treated cells (an irreversible inhibitor of mitosis) to assure that wound closure was due, in fact, to cell migration and not the result of cell proliferation.

Remarkably, similar to that observed for the oncogenic mutant RET C634R, both pools transfected with RET new variants, RET T636M and RET C515W, showed a migration capacity (measured by faster wound closure) notably higher than that observed for the RET-WT pool (Figure III.2).

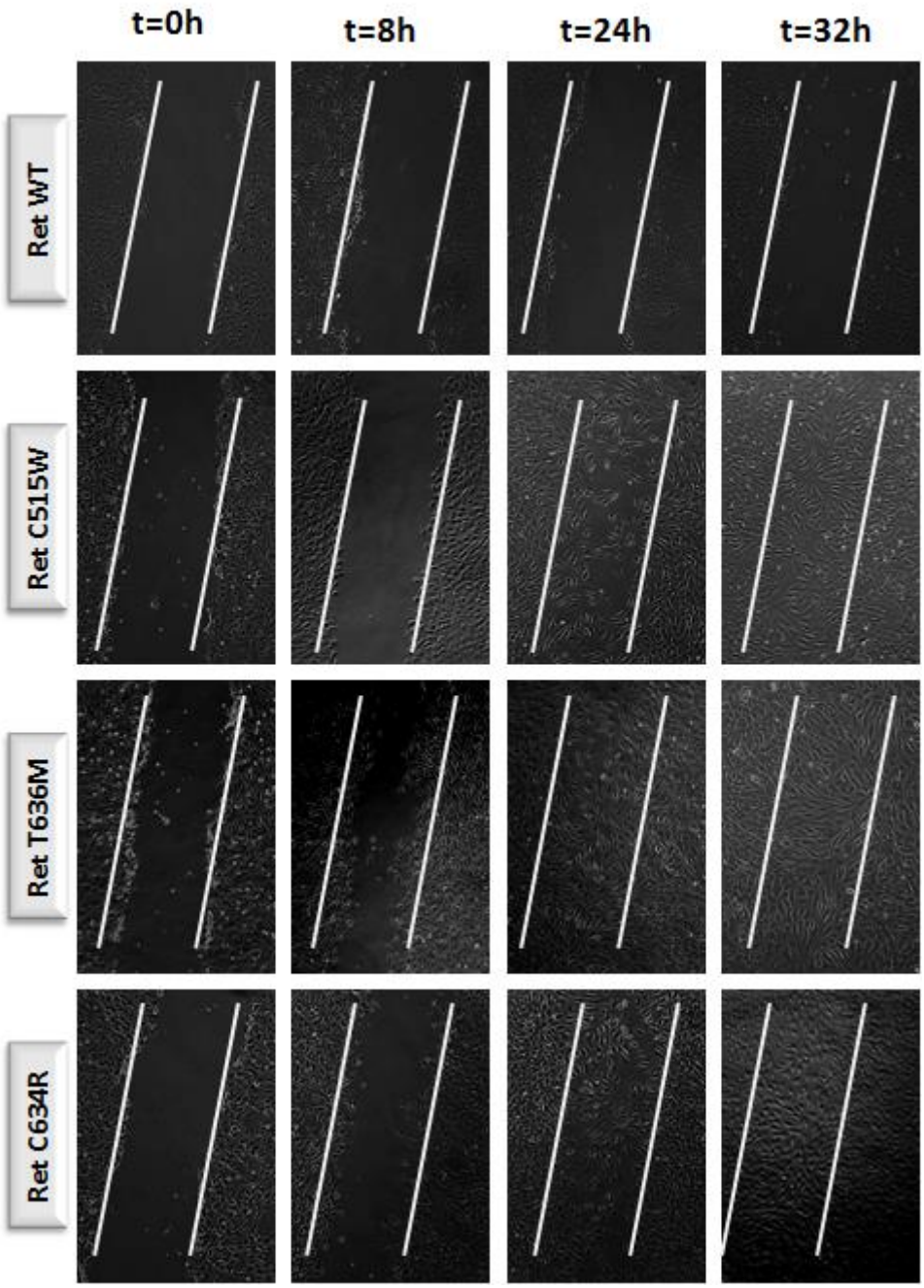


Figure 9 - *In vitro* Wound Healing assay. Wound closure was photographed when the scrape wound was introduced (t=0 hours) and at designated times after wounding (t=8hours, t=24hours, t=32hours). Phase microscopy (original magnification: 200x).

Discussion

The RET proto-oncogene encodes a tyrosine kinase receptor expressed in tissues derived from the neural crest. Germline activating mutations of RET predispose inheritance of FMTC or MEN 2 cancer syndromes. Germline testing of RET can be used to distinguish sporadic MTC cases from hereditary MTC. RET genetic testing to identify at-risk family members is considered the standard of care in MEN 2 families. In fact, since RET has been recognized as a causative oncogene for MEN 2 syndromes, considerable research has been made to identify and characterize RET germline mutations and their association with disease onset and aggressiveness (Fazioli et al., 2008).

The clinical course of hereditary MTC varies from extremely indolent tumors, which can go unchanged for years, to extremely aggressive variants, associated with a high mortality rate (Mian et al., 2012). Clinical phenotype, age of onset, and aggressiveness of MTC are related to the oncogenic potential of the RET proto-oncogene mutation involved in each case. In fact, several risk stratification strategies based on mutation oncogenicity have been developed to guide clinicians in making medical management recommendations such as age of genetic testing and prophylactic thyroidectomy. Nevertheless, while genotype-phenotype correlations are well-established for most disease causing RET mutations, a number of rare or novel RET mutations have been also described. While some of these proved to be devoid of clinical significance, others represent mutations leading to FMTC or MEN2 phenotypes, or sometimes constitute low penetrance or modifying alleles that confer only a modest risk of developing MTC. The functional and clinical significance of a given genetic variant should, therefore, be determined, using several approaches such as *in silico* analyses, association studies, and functional assays.

The main objective of this work was to evaluate the oncogenic potential of two novel germinal variants associated with MTC cases. These variants correspond to missense alterations in codons 515 and 636 (RET C515W and RET T636M located in exons 8 and 11, respectively), which result in amino acid substitutions in the extracellular region of the RET protein.

RET T636M variant was identified in a 79 year old woman with no familial evidence of MEN2A/2B or FMTC, who underwent thyroidectomy due to a unifocal MTC. RET C515W variant was identified in a 60 year old woman who underwent total thyroidectomy due to a multifocal MTC. Notably, this patient was found to be homozygous for this new RET variant. This variant, however, was also found in heterozygosity in a proband's relative who also developed MTC at age 63.

To predict the degree of alteration determined by a missense point mutation on the biological activity of the encoded protein, functional *in silico* analysis was performed by using several software tools. RET T636M variant was mostly predicted to have no phenotypic effect, despite T636 being an evolutionary conserved residue. RET C515W, on the other hand, was mostly predicted to be possibly damaging (it may affect protein function and/or structure). This analysis, although useful, is nonetheless somewhat limited. Bioinformatics prediction tools may be valuable as screening tools for identifying alleles of high pathogenic potential for molecular and disease association studies. However, because the error rates associated with predictions are still high, current algorithms do not supplant the need for *in vitro* or *in vivo* studies (Wang et al., 2012).

In vitro functional experimental approaches were therefore developed in order to evaluate the oncogenic potential of these two variants. By expressing the new RET variants in non-transformed NIH3T3 mouse fibroblasts, and by comparing their effect with wild-type RET, we were able to observe an increase of cell growth and proliferation (Figures III.2 and III.3), the loss of contact inhibition (Figure III.1) and an increase in cell migration rate (Figure III.4). All these are common characteristics of tumoral cells, indicating that these new RET variants hold some relevant transforming potential. This potential was, nevertheless, low when compared to MEN2A-causing C634R RET mutant, used as positive control.

Germline point mutations of RET which are responsible for autosomal dominant MEN 2 cancer syndromes and FMTC fall into two main groups: those affecting codons in the extracellular domain and those affecting the tyrosine kinase domain. These point mutations in RET induce a ‘‘gain-of function’’ effect in the encoded protein. Constitutive dimerization is the molecular mechanism of the activation of RET molecules carrying mutations affecting extracellular cysteines. In the wild-type

receptor, these cysteines form intramolecular disulfide bonds, stabilizing the receptor in its monomeric ligand-unbound conformation. When mutations lead to the loss of a cysteine residue, the resulting unpaired cysteine is free to form an activating intermolecular bridge with another mutated receptor (Arighi et al., 2005). This generates covalently bound receptors that are constitutively active due to receptor dimerization, irrespective of ligand binding (Prazeres et al., 2011b).

The most frequently identified RET mutations in MEN 2 affect cysteines in the extracellular cysteine-rich region (primarily residues between Cys515 and Cys634) (Wagner et al., 2012). Although the RET T636M variant doesn't involve directly a cysteine residue, it can induce a conformational change in the protein that may affect an intramolecular disulfide bond attained by a cysteine located near this variant. This could lead to an impact on RET structure and activation. Notably, RET T636M variant is located in the vicinity of several codons that, when mutated, are associated with more aggressive phenotypes.

On the other hand, RET C515W variant directly involves a cysteine residue. Hence, the replacement of a normal cysteine with any amino acid, can lead to loss of intramolecular bonds leaving an unpaired cysteine available for intermolecular interactions with other mutant RET proteins. Although the molecular mechanisms of activation are similar, cysteine RET mutations also vary in their impact on RET activation. In general, mutations located closer to the RET transmembrane domain have greater transforming ability and are linked to increased risk of more aggressive MEN 2 disease (Wagner et al., 2012). In addition to RET C515W variant, several RET variants in exon 8 (such as Cys515Ser, Gly533Cys and Glu515Lys) have been demonstrated to display oncogenic features. In fact, exon 8 of RET has been suggested to be an underestimated hotspot of RET variants with an oncogenic potential (Muzza et al., 2010).

Individuals with weakly transforming mutations in RET gene could require a second germline or somatic mutation to develop a more aggressive phenotype that results in clinical expression of the disease. This was firstly hypothesized to be the case of patient B who was found to be a homozygous carrier of C515W variant. This hypothesis, however, was set aside when a heterozygous carrier for this mutation was also found to express the disease, suggesting that one mutated allele is sufficient to

develop MTC. Whether the transforming ability of a second weakly transforming RET alteration would account to a more aggressive phenotype remains to be determined. External factors, such as lifestyle and environment added to late onset and low penetrance are likely to contribute to variations in the clinical expression of the disease, making it hard to assign the effect of the mutation itself.

In sum, these two new variants have shown a low transforming potential through *in vitro* functional assessments, consistent with the late onset and low aggressiveness observed in the patients.

Future perspectives

In order to better understand the role of the variants Ret C515W and Ret T636M in MTC development,, it would be important to perform additional *in vitro* functional studies, such as to assess the degree of activation of the novel RET variants and their ability to induce anchorage-independent cell growth.

Colony forming capacity in semisolid media assessment (soft-agar assay) has been considered to be fundamental in cancer biology because it has been connected with features of tumor cell aggressiveness *in vivo* such as tumorigenic and metastatic potentials (Mori et al., 2009). Non-transformed adherent cells must attach to a solid surface before they can divide and they fail to grow when suspended in a viscous fluid or gel (e.g. agar or agarose). Nevertheless, when cells are transformed, they are able to grow in a viscous fluid or gel and become anchorage-independent.

Another important assay would be to evaluate the degree of RET tyrosine kinase activation induced by the newly identified variants. The ligands of the RET receptor are growth factors belonging to the glial cell line-derived neurotrophic factor (GDNF) family. In the absence of the ligand, the RET receptor tyrosine kinase is monomeric, unphosphorylated, and, hence, inactive. Binding of the ligand to the extracellular domain of the RET tyrosine kinase induces receptor dimerization and auto-phosphorylation, creating intracellular binding sites for signaling proteins with the subsequent activation of multiple signaling pathways receptor(Figlioli et al., 2013). Hence, tyrosine auto-phosphorylation of RET can be a measure of the degree of activation of RET receptor tyrosine kinase.

A more complete functional characterization of these two new variants will not only contribute to the understanding of the molecular mechanisms behind the development of MTC in these families, but will also provide useful information to optimize the diagnostic and clinical management of hereditary MTC. In this context, it might also be important to consider extending the genetic screening to all first degree relatives of positive carriers in these families.

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Appendix

Solutions, buffers and reaction conditions for the experimental work:

Reagents	Final concentration (final 20 µl reaction)
Random primers (Roche)	0,015 µg/µL
dNTPs (GE Healthcare Life Sciences)	0,5 mM
2µg of total RNA was used in each reaction	
H_2O to a final volume of 12,9 µl	
Samples were incubated at 65°C for 10 min and then cooled to 4°C	
7,1 µl of reverse transcription mix (table 2) was added	

Supplementary Table 1 - cDNA synthesis (RNA denaturation and primer annealing)

Reagents	Final concentration (final 20 µl reaction)
DTT (Invitrogen™)	0,01M
RNAse Out (Invitrogen™)	1 U/µL
SuperScript II (Invitrogen™)	10 U/µL
First strand Buffer (Invitrogen™)	1x

Supplementary Table 2 - cDNA synthesis (Reverse transcription)

Stage	Temperature (ºC)	Time
cDNA synthesis	42	1 hour
Inactivation	70	15 minutes
Cooling	4	∞

Supplementary Table 3 – Conditions of cDNA synthesis (Reverse transcription)

Reagents	Final concentration
Primer Foward	0,4 pmol/ µL
Primer Reverse	0,4 pmol/ µL
Taq DNA polymerase (Invitrogen™)	0,03 U/µL
PCR Buffer (Invitrogen™)	1x
MgCl2 (Invitrogen™)	1,5mM
dNTPs (GE Healthcare Life Sciences)	0,2mM
2 µl of cDNA was used in 25 µl of reaction mix	

Supplementary Table 4 - PCR amplification of RET cDNA (exons 8 to 11)

Stage	Temperature (ºC)	Time	Cycles
Initial denaturation	95	5 minutes	1
Denaturation	95	30 seconds	
Annealing	56	30 seconds	34
Elongation	72	30 seconds	
Final elongation	72	7 minutes	1
Inactivation	4	10 minutes	
Cooling	12	∞	

Supplementary Table 5 – Conditions of PCR amplification of RET cDNA (exons 8 to 11)

Solutions	Final concentration
Sample buffer	1 U/µL Benzonase (Sigma); 5 mM $MgCl_2$; 200 mM Tris-HCl, pH 6.8; 5% glycerol; 2% SDS; 100mM DTT; 0,1% (v/v) Bromophenol blue 0,1%
SDS-page running gel	Running Gel Buffer (375 mM Tris/HCl pH 8.8); 10% Acrylamide (BioRad); 0,1% (v/v) SDS; 0,05% (v/v) TEMED; 1% (v/v) APS
SDS-page stacking gel	Stacking Gel Buffer (62,5 mM Tris/HCl pH 8.8); 4% Acrylamide (BioRad); 0,1% (v/v) SDS; 0,05% (v/v) TEMED; 1% (v/v) APS
Blott buffer	25 mM Tris-HCl (pH 7.6) , 192 mM glycine, 20% metanol, 0.03% SDS
Destain solution	10% (v/v) acetic acid; 45% (v/v) Methanol
TBST	50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0,05% (v/v) Triton X-100 (Sigma)
TBST milk	10% (w/v) milk powder in TBST buffer
ECL solution	100 mM Tris pH 8.8; 1,875 mM luminol; 225 µM cumaric acid; 0,05% (v/v) hydrogen peroxide

Supplementary Table 6 - Buffers and final concentrations for Western Blott

Reagents	Final concentration
PBS	10 mM Na2HPO4, 1.8 mM KH2PO4, 137 mM NaCl, 2.7 mM KCl pH7.4
Propidium iodide	0,1 mg/mL
RNAse A (Invitrogen™)	2mg/mL

Supplementary Table 7 - Propidium iodide solution for cell cycle analysis