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Genetic susceptibility to thyroid cancer: contributions of *RET* polymorphisms

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Resumo alargado

A glândula da tiróide é o maior órgão do corpo humano exclusivamente endócrino. Localiza-se na parte anterior do pescoço, em frente à traqueia e abaixo da laringe, é um órgão bastante vascularizado e em forma de borboleta. Histologicamente a glândula da tiróide é constituída por folículos formados por epitélio simples de células foliculares produtoras das hormonas T3 e T4, nos interstícios dos folículos localizam-se as células parafoliculares produtoras da hormona calcitonina. As hormonas T3 e T4 produzidas por esta glândula regulam a taxa do metabolismo e afectam o aumento e a taxa funcional de muitos outros sistemas do organismo, já a hormona calcitonina possui um papel importante na homeostase do cálcio. Como qualquer outro órgão também a tiróide está sujeita a fenómenos de carcinogénese, o cancro da tiróide tem uma incidência anual de 212.000 casos por todo mundo, este número tem vindo a aumentar nos últimos anos devido, provavelmente, aos progressos nas técnicas de diagnóstico e ao efeito dos factores ambientais.

O cancro da tiróide tem uma etiologia multifactorial resultante da interacção de factores genéticos e ambientais. Dependendo do tipo de células a partir das quais o cancro se desenvolve, o cancro da tiróide pode ser dividido em dois grandes grupos, os carcinomas medulares da tiróide (MTC) e os carcinomas diferenciados da tiróide (DTC). A última categoria (DTC) abrange o carcinoma folicular da tiróide (FTC) e o carcinoma papilar da tiróide (PTC), ambos originam-se a partir das células foliculares e representam a maioria dos tumores desta glândula. Apesar da grande incidência, de um modo geral, estes subtipos apresentam bom prognóstico e boa taxa de sobrevivência. Os DTC podem evoluir para um estado indiferenciado com prognóstico muito mais reservado designado carcinoma anaplásico da tiróide (ATC). O MTC tem origem nas células parafoliculares da tiróide. Os mecanismos moleculares subjacentes ao desenvolvimento do cancro da tiróide têm sido amplamente estudados, e o proto-oncogene *RET* tem sido desde há muito associado a este cancro, desde rearranjos cromossómicos no caso do PTC a mutações pontuais a nível germinativo e somático no MTC. Este proto-oncogene localiza-se no braço longo do cromossoma 10 e é composto por 21 exões, codifica um receptor transmenbranar tirosina-cinase com o mesmo nome do gene. O receptor RET é expresso nas células precursoras da crista neural e do tacto urogenital, sendo necessário na maturação de várias linhagens celulares do sistema nervoso periférico bem como na morfogénese dos rins e na espermatogénese. Mutações que levam à perda de função deste receptor têm sido associadas à doença de Hirchsprung, doença poligénica associada à ausência de neurónios entéricos no tracto gastrointestinal. Por outro lado, mutações que levam ao ganho de função deste receptor estão associadas ao cancro, em particular ao cancro da tiróide.

Um *locus* é considerado polimórfico quando um ou mais dos seus alelos raros têm uma frequência de pelo menos 1% numa população. Um conjunto de marcadores genéticos ou polimorfismos que se transmitam como uma unidade através de gerações é designado de haplótipo. Variantes polimórficas comuns têm vindo a ser associadas com o fenótipo de várias doenças complexas, modificando assim o risco de ocorrência das mesmas. Igualmente, nos últimos anos, os polimorfismos do proto-oncogene *RET* têm sido estudados de modo a determinar se estas variantes polimórficas podem representar alelos de baixa penetrância que predisponham para doenças associadas ao *RET* (como o PTC, o MTC familiar e esporádico e a doença de Hirschprung). No caso da tiróide, a maioria dos estudos existentes diz respeito ao MTC, de um modo geral os polimorfismos que têm mostrado uma maior prevalência nas populações de doentes relativamente à população geral são: o G691S, o L769L, o S836S e o S904S. Estes localizam-se nos exões 11, 13, 14 e 15, respectivamente, e albergam os *hot spots* mutacionais para o MTC. Contudo, é clara a controvérsia entre os diversos estudos no que respeita à influência dos polimorfismos na susceptibilidade para o cancro da tiróide.

Com este trabalho, pretendeu-se investigar a associação dos polimorfismos G691S, L769L, S836S e S904S, isoladamente ou em conjunto, com o aumento do risco para o desenvolvimento do cancro da tiróide, em particular dos DTC na população portuguesa. Paralelamente pretendeu-se avaliar a associação destes polimorfismos com 4 parâmetros clínicos: o subtipo de cancro, o género, a idade de diagnóstico e o tamanho do tumor no diagnóstico. Para alcançar este objectivo, fez-se um estudo de associação caso-controlo. Para além de uma população controlo, foi necessária a recolha de uma população de doentes com PTC ou FTC e respectivos dados clínicos relevantes. A população de doentes consistiu em 282 indivíduos, portugueses e caucasianos, cedidos pelo Instituto Português de Oncologia de Coimbra. A população controlo foi formada por 245 voluntários, também portugueses e caucasianos, não relacionados, recrutados entre pessoal da Faculdade e dadores de sangue do Instituto Português de Sangue da Região Centro.

Após a obtenção do consentimento informado, foi recolhida uma amostra de sangue periférico de cada indivíduo. A genotipagem dos indivíduos do grupo de doentes e do grupo controlo iniciou-se pela extracção do DNA a partir de uma amostra de sangue periférico pelo método de *salting-out* adaptado, seguiu-se a quantificação desse mesmo DNA de forma a obter informação sobre a concentração e nível de pureza da amostra de DNA. Em seguida, fez-se a amplificação por PCR dos exões, onde se encontram os quatro polimorfismos estudados. Para conseguir distinguir os genótipos dos indivíduos para os vários polimorfismos fizeram-se incubações com enzimas de restrição, dado que que todos os polimorfismos criam ou eliminam um local de restrição para uma endonuclease específica. Para garantir a correcta genotipagem pelo método anterior, procedeu-se à sequenciação de DNA de 3 indivíduos representativos dos 3 genótipos para cada um dos polimorfismos estudados. Assim, foi então possível a formação de uma base de dados dos genótipos das duas populações, permitindo a

análise estatística das mesmas. Nesta análise, avaliou-se o equilíbrio de Hardy-Wenberg para a população controlo, bem como o desequilíbrio de ligação (LD) entre os vários polimorfismos, seguiu-se a comparação das distribuições alélicas, genotípicas e haplotípicas entre controlos e doentes. Para a avaliação dos vários parâmetros clínicos em estudo, fizeram-se subdivisões da população de doentes de acordo com cada um dos parâmetros e compararam-se as mesmas.

Os resultados obtidos permitiram-nos observar que a população portuguesa, usada como controlo neste estudo, se assemelha à população europeia no que diz respeito à distribuição genotípica dos polimorfismos estudados, com excepção do polimorfismo L769L. Verificou-se um forte LD entre as variantes polimórficas G691S e S904S, sugerindo a sua co-segregação conjunta, tal como verificado em outros estudos populacionais. Pelas várias comparações realizadas, este estudo permitiu sugerir que o polimorfismo S836S parece estar associado com um risco aumentado de desenvolver DTC. Verifica-se também, uma possível associação entre os genótipos heterozigóticos dos polimorfismos G691S e S904S e a idade de desenvolvimento do DTC e, isoladamente, o genótipo heterozigótico do polimorfismo G691S parece ter associação com o tamanho do tumor. O haplótipo GGTC parece estar associado com uma maior susceptibilidade para o DTC em particular em idades mais avançadas, diagnóstico depois dos 45 anos. Contudo, com o ajuste para comparações múltiplas, muitos destes resultados não se mostraram estatisticamente significativos. Assim, estes resultados necessitam de ser confirmados em outros estudos com uma maior amostra para se poder reavaliar o papel destas variantes na susceptibilidade para o DTC.

Palavras-chave

Cancro da tiróide; carcinoma papilar da tiróide; carcinoma folicular da tiróide; gene RET; polimorfismos.

Abstract

Thyroid cancer is the most common malignancy of the endocrine system, represents more than 1% of all malignancies and has an estimated annual incidence of 212,000 cases worldwide. The term differentiated thyroid carcinoma (DTC) comprises the subtypes papillary thyroid carcinoma (PTC) and follicular thyroid carcinomas (FTC), these subtypes represent the two most common subtypes of thyroid cancer (approximately 80% and 10% respectively). Despite its incidence DTCs have a good prognosis with relatively few metastases and deaths associated. The polymorphisms (variants in DNA sequence among individuals that have a frequency of at least 1% in a population) of *RET* proto-oncogene have been studied in different populations for association with susceptibility to thyroid cancer, but with inconsistent findings mainly in DTC. To clarify the contribution of single locus or haplotypes (polymorphisms that are transmitted through generations as a unit) of *RET* polymorphisms to genetic susceptibility to DTC among Portuguese patients, we conducted a case–control study by analyzing four well-characterized *RET* polymorphisms (G691S, L769L, S836S and S904S).

To achieve this aim, the *RET* polymorphisms were genotyped and haplotype frequencies were estimated in a population of 282 individuals with DTC and in a control population of 254 individuals. Allele, genotype and haplotype distributions were compared among cases and controls. Patient population was subdivided according to several clinical parameters and allele, genotype and haplotype distributions were compared among the subgroups.

The single locus analysis showed an overrepresentation of the S836S polymorphism in patients when compared to controls. Also the heterozygous genotypes of the G691S/S904S polymorphisms were overrepresented in cases diagnosed after the age of 45 years and the heterozygous genotype of G691S polymorphism revealed an overrepresentation in patients with tumors larger then 10mm of diameter at diagnosis. The haplotype analysis showed an overrepresentation of GGTC haplotype in patients particularly in those diagnosed after the age of 45 years.

In conclusion, our data suggest that the S836S polymorphism may be associated with increased risk of DTC. Also the heterozygous genotype of the G691S/S904S polymorphisms seems to be associated with age of onset of DTC and additionally the heterozygous genotype of G691S polymorphism appeared to be in association with tumor size. Finally, one haplotype appears to be associated with increased risk of DTC particularly in those developed in later age (after the age of 45 years). These findings need to be confirmed by larger studies in order re-evaluate the role of these variants in the susceptibility to DTC.

Keywords

Thyroid cancer; Papillary Thyroid Carcinoma; Follicular Thyroid Carcinoma; *RET* gene; polymorphisms.

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List of abbreviations

AKAP9	A-kinase anchor protein 9
AKT	Also known as Protein Kinase B (PKB)
AKT1	V-akt murine thymoma viral oncogene homolog 1
ARAF	V-raf murine sarcoma 3611 viral oncogene homolog
ARTN	Artemin
ATC	Anaplastic thyroid carcinoma
ATP	Adenosine triphosphate
bp	Base pairs
BRAF	V-raf murine sarcoma viral oncogene homolog B1
BSA	Bovine serum albumin
CCDC6	Coiled-coil domain containing 6
CRAF	Raf-1 murine leukemia viral oncogene homolog 1
CTNNB1	Catenin (cadherin-associated protein) beta 1
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
DTC	differenciated thyroid carcinoma
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinase
FMTC	Familial MTC
FTC	Follicular thyroid carcinoma
GDNF	Glial cell line-derived neurotrophic factor
GFL	GDNF family of ligands
GFRα	GDNF receptor alpha
Gly	Glycine
H-RAS	Harvey rat sarcoma viral oncogene homolog
K-RAS	Ras2 Kirsten rat sarcoma viral oncogene homolog
LD	Linkage disequilibrium
Leu	Leucine
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
MEN-2	Multiple endocrine neoplasia type-2
MEN-2A	Multiple endocrine neoplasia type-2A
MEN-2B	Multiple endocrine neoplasia type-2B
MTC	Medullary thyroid carcinoma
NCOA4	Nuclear receptor coactivator 4
NGF	Nerve growth factor

NIH 3T3 Mouse embryonic fibroblast cell line
N-RAS Neuroblastoma RAS viral (v-ras) oncogene homolog
NRTN Neurturin
NTRK1 Neurotrophic receptor-tyrosine kinase
OD Optical density
PAX8 Paired box gene 8
PCR Polymerase chain reaction
PI3K Phosphatidyl-inositol-3'-kinase
PIK3CA PI3K subunit alpha
PPARγ Peroxisome proliferator-activated receptor gamma
PSPN Persesephin
PTC Papillary thyroid carcinoma
PTEN Phosphatase and tensin homolog
RAS Rat sarcoma
RBC Red blood cells
RET Rearranged during transfection
RET43 RET isoform with 43 amino acids in carboxy - terminal tail
RET51 RET isoform with 51 amino acids in carboxy - terminal tail
RET9 RET isoform with 9 amino acids in carboxy - terminal tail
RNA Ribonucleic acid
rpm Rotations per minute
SD Standard deviation
Ser Serine
SNP Single nucleotide polymorphism
T3 Triiodothyronine
T4 Thyroxine
TK Tyrosine - kinase
TRH Thyrotropin-releasing hormone
TRK Tropomyosin-related kinase
TSH Thyroid-stimulating hormone
UV Ultraviolet

1. Introduction

1.1. Thyroid Gland

1.1.1. Anatomy and histology

The thyroid gland is the largest organ, in the body, uniquely qualified to produce hormones. The thyroid gland weighs approximately 20 to 25 g, is located in the anterior neck at the level of the fifth cervical vertebra to the first thoracic vertebra, in front of the trachea, and immediately below the larynx. It is covered by the neck muscles and its fascia. It has a butterfly-shape and is constituted by two lobes joined together by an isthmus (narrow band of thyroid tissue). The thyroid is a very vascularized organ rich in blood and lymph capillaries and therefore presents a more reddish color than the surrounding tissues [1, 2].

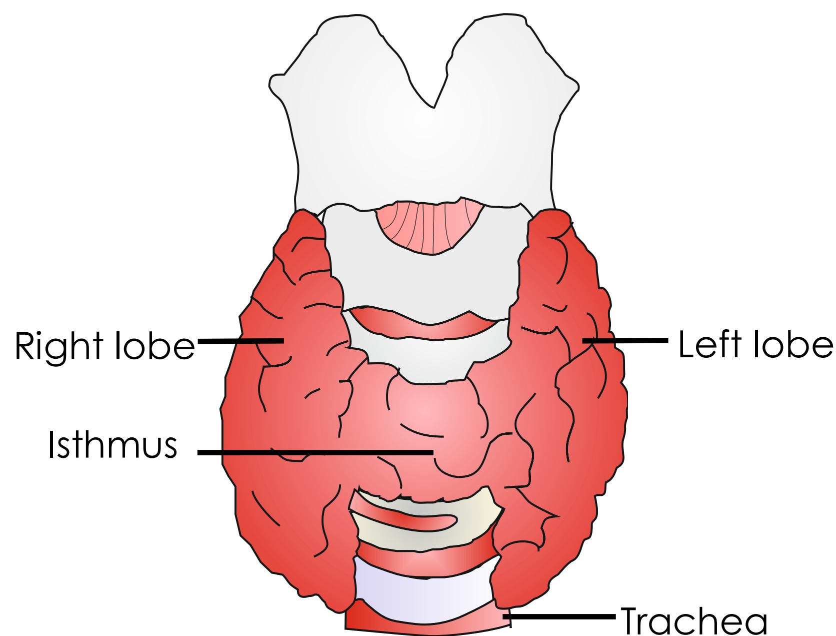

Figure 1 – Frontal view of the thyroid gland. The thyroid gland is located in the anterior neck in front of the trachea, and immediately below the larynx, it has a butterfly-shape and is constituted by two lobes joined together by an isthmus.

Histologically the thyroid gland is composed by numerous thyroid follicles, which are spherical sacs filled with colloid rich in a protein called thyroglobulin to which thyroid hormones are bound. Thyroid follicles are lined by a single layer of thyroid epithelial cells, the follicular cells. In the soft connective tissue that separates and surrounds the thyroid follicles lie the parafollicular or C cells [3].

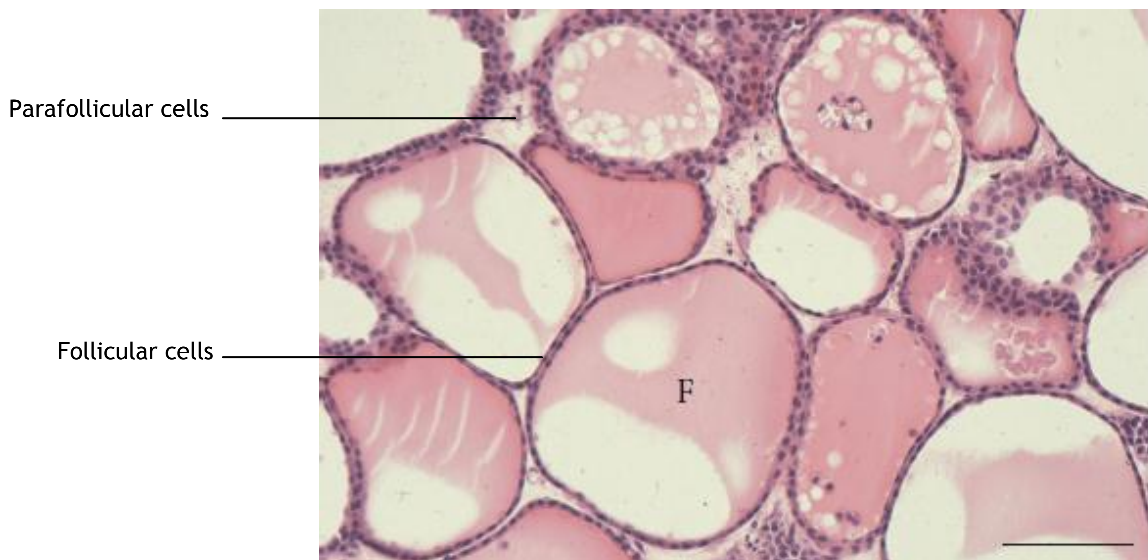

Figure 2 - Histology of the thyroid gland. Histologically the thyroid gland consists in many spherical thyroid follicles containing thyroglobulin. Parafollicular cells are in the tissue between the thyroid follicles. F- Thyroid follicle. *Adapted from Toda et al., 2011* [4].

1.1.2. Thyroid hormones

Briefly the function of the thyroid gland is to secrete an appropriate amount of the thyroid hormones. The thyroid gland produces two main types of hormones that are structurally related: thyroxine (named T4 since it carries 4 iodine atoms) and triiodothyronine (named T3 since it carries 3 iodine atoms). Under normal circumstances T4 constitutes approximately 90 percent of the hormones produced in the thyroid gland [5]. However, T3 is a much more active hormone, and the majority of the T4 produced by the thyroid is converted into T3 in the liver and kidneys [6].

T3 and T4 are produced and secreted by follicular cells and are stored in the thyroid follicles. The secretion of T3 and T4 is increased by the hypotalamic thyrotropin-releassing hormone (TRH), which stimulates release of thyroid-stimulating hormone (TSH), by the anterior pituitary, in response to low T3 and T4 levels, low metabolic rate, pregnancy, cold and high altitudes. High T3 and T4 levels inhibit TRH and TSH secretions; high iodine level suppresses the secretion of these hormones [7]. Almost all body cells have receptors for thyroid hormones, so T3 and T4 exert their effects throughout the body. The principal actions of T3 and T4 are to increase basal metabolic rate, increase use of glucose and fatty acids for ATP production, stimulate synthesis of proteins, enhance cholesterol excretion, accelerate body growth and contribute to development of the nervous system [7].

In addition to T3 and T4, the thyroid gland also produces calcitonin. Calcitonin is a polypeptide hormone produced and secreted by the parafollicular cells, which helps regulate

calcium homeostasis by diminishing the concentration of Ca^{2+} in body fluids, when Ca^{2+} levels are elevated. Taking into account this effect, calcitonin is a direct antagonist of the hormone produced by the parathyroid glands (parathyroid hormone). The target of calcitonin is the skeleton where it inhibits the action of osteoclasts (cells that break down bone extracellular matrix), so the level of Ca^{2+} in the blood decreases. Consequently, calcitonin promotes calcium deposition and bone formation by stimulating osteoblast activity [7]. Excessive blood levels of Ca^{2+} (approximately 20% above normal) act as a humoral stimulus for the release of calcitonin in the blood, while the low levels of Ca^{2+} inhibit the secretory activity of the parafollicular cells. Calcitonin appears to be important only in childhood, when the skeleton has an accentuated growth and the bones are changing in mass, size and shape [3].

1.2. Thyroid cancer

Thyroid cancer is the most common malignancy of the endocrine system [8]. Thyroid cancer represents more than 1% of all malignancies and has an estimated annual incidence of 212,000 cases worldwide. This number has increased quickly in recent years, probably due to the improvement of diagnostic techniques and to the effect of environmental factors [9].

Thyroid cancer has a multifactorial etiology resulting from the interaction of genetic and environmental factors in individuals at risk. Regarding the environmental level the major parameters to take into account are: the exposure to ionizing radiations, age at the time of the exposure, presence of a previous history of benign thyroid disease, role of the dietary iodine intake, role of the body mass index, and role of hormonal factors [10]. Thyroid cancer usually develops in thyroid nodules. Thyroid nodules are common, principally in adults with the increase of age, and most are benign. They can be detected by palpation and by imaging, the clinical challenge lies in the rapid and accurate identification of those nodules that hide cancer [11].

Thyroid cancer comprises two main sets of neoplasias, depending on the type of cell affected by malignant transformation. When the cancer develops from follicular cells it gives rise to follicular thyroid carcinoma (FTC) or papillary thyroid carcinoma (PTC), representing the majority of thyroid tumors, and the anaplastic thyroid carcinoma (ATC) is the undifferentiated type of carcinoma derived from follicular cells. Only a small part of cancers originates from parafollicular cells giving rise to medullary thyroid carcinoma (MTC) [9].

1.2.1. Follicular-cell-derived carcinoma

Follicular-cell-derived tumors include benign adenomas and well differentiated (papillary or follicular), poorly differentiated (insular), and undifferentiated (anaplastic) carcinomas.

Between them, PTC and FTC represent the two most common subtypes, representing 80-85% and 10-15% respectively [12, 13]. PTC and FTC are termed differentiated thyroid carcinoma (DTC)[14].

These subtypes, in general, display a good prognosis with relatively few metastasis and deaths[15], contrasting with the insular and anaplastic carcinomas, that though less frequent (approximately 5% of all thyroid carcinomas) show a poor prognosis. PTC is defined on the basis of the histological pattern and distinct nuclear features, that have ground glass appearance and longitudinal grooves with cytoplasm invaginations [16]. PTC may be sporadic (95%) or familial (5%) [17], its incidence is remarkably high in developed countries, and metastasizes to local lymph nodes [18]. PTC has a sex ratio female:male of around 3:1, predicting the influence of hormonal factors which are still unknown. Exposure to ionizing radiation is the only established environmental factor related to PTC [19].

There are numerous histopathologic variants of PTC according to a set of distinctive nuclear features (include nuclear enlargement and irregularity, overlapping, clearing, grooves, and pseudo-inclusions). The criteria used to subclassify PTC are not clearly defined, so classifying PTC is a major problem. The well-established subtypes include: classical variant, follicular variant, papillary microcarcinoma (micro-PTC), oncocytic variant (Hurthle cell), tall cell variant, clear cell variant, solid variant, cribriform-morular variant, columnar cell variant, diffuse sclerosing variant and macrofollicular variant [20, 21].

Emphasizing the micro-PTC, this subtype is defined as a papillary carcinoma that measures in maximum 1 cm that belongs to the low-risk well-differentiated PTC. Micro-PTCs are very common and is important distinguish between micro-PTC associated with clinically larger and significant PTC and an incidental micro-PTC found after thyroidectomy performed for other indications or during thyroid ultrasound. In the first case micro-PTC have been considered to represent dissemination of the larger tumor, on other hand incidental micro-PTC has an outstandingly good prognosis and there is nearly no risk of recurrence or death [20, 22-24].

What distinguishes the FTC is the absence of nuclear morphological features that define the PTC. The majority of the tumors is encapsulated, and may be composed of follicles or follicular cells arranged in follicular, solid or trabecular patterns. FTC compared to PTC tends to be more aggressive, produces distant metastases, and presents a more balanced sex ratio (about 2:1, female:male). FTC has been associated with a deficiency in iodine intake and is more frequent in developing countries [25].

1.2.2. Medullary thyroid carcinoma

Medullary thyroid carcinoma (MTC) originates from the parafollicular cells and represents about 3% to 5% of all thyroid cancers [13]. Around 75% of MTC occur sporadically and the

other 25% are hereditary. The hereditary MTC is part of a syndrome known as multiple endocrine neoplasia type-2 (MEN-2) that has an autosomal dominant model of inheritance. MEN-2 can be divided in three clinically distinct forms: MEN-2A, MEN-2B, and familial medullary thyroid carcinoma (FMTC).

MTC is histopathologically much more homogeneous than DTC. These carcinomas have a poorer prognosis as more than 50% of the patients develop local metastases to cervical and mediastinal nodal groups, and approximately 20% have distant metastases to the lung, liver, or bone at time of diagnosis [18]. Patients with MTC have a 10 year rate mortality that varies between 13.5% and 38% [26].

1.2.3. Molecular genetics of thyroid cancer

Thyroid cancer initiation and progression includes a set of genetic (as somatic mutations) and epigenetic alterations (as aberrant gene methylation and micro RNA dysregulation) as occurs in other cancers. Somatic mutations represent most of the information obtained until now; these mutations have a role in the early carcinogenic process and are vital for the development of cancer.

The critical genes involved in thyroid cancer are usually mutated under two types of molecular mechanisms: i) point mutation, which is a result of single nucleotide change within the DNA chain; ii) chromosomal rearrangement, which is a genetic abnormality with breakage and fusion of parts of the same or different chromosomes, this mechanism encompass several different classes of events (deletions, duplications, inversions and translocations). These two molecular mechanisms have been associated with specific etiologic factors involved in thyroid cancer development [13].

The majority of the mutations in thyroid carcinomas involve the effectors of the mitogen-activated protein kinase (MAPK) pathway and the PI3K–AKT pathway. The MAPK pathway is a crucial intracellular cascade that regulates cell growth, differentiation, apoptosis and survival, and when aberrantly activated, tumorigenesis [27, 28]. These pathways are affected by mutations in genes encoding the transmembrane receptor tyrosine kinase (TK) RET and NTRK1 and intracellular signal transducers BRAF and RAS. In the case of FTC there is another quite common event in addition to *RAS* mutations, this event is PAX8/PPAR-γ rearrangement. Progression and dedifferentiation of thyroid carcinomas involves other mutations that affect the PI3K–AKT pathway and other cell signaling pathways [29, 30]. Table 1 compiles the mutational profiles for the major types of thyroid cancer. The genetic alterations of the *RET* gene are described in more detail in the section intended for this gene.

Table 1 - Thyroid cancer types and correspondent mutational profiles [13].

Features	Papillary carcinoma	Follicular carcinoma	Anaplastic carcinoma	Medullary carcinoma
Cell type	Follicular	Follicular	Follicular	Parafollicular
Prevalence %	80 - 85	10 - 15	1 - 2	3 - 5
Frequency of familial forms %	5	5	0	15 – 30
10 years survival %	95 - 98	90 - 95	< 10	60 - 80
Common mutations (prevalence %)	*BRAF* (40–45) *RAS* (10–20) RET/PTC (10–20) *TRK* (<5)	*RAS* (40–50) PAX8/PPARγ (30–35) *PIK3CA* (<10) *PTEN* (<10)	*TP53* (50–80) *CTNNB1* (5–60) *RAS* (20–40) *BRAF* (20–40) *PIK3CA* (10–20) *PTEN* (5–15) *AKT1* (5–10)	Familial forms: *RET* (>95) Sporadic: *RET* (40–50) *RAS* (25)

1.2.3.1. *BRAF* mutations

The *RAF* family encodes serine/threonine kinases that are key signal transducers of diverse extracellular stimuli and MAPK signaling pathway [27, 28].

The *RAF* family is composed of three isoforms, *ARAF*, *CRAF* (*RAF-1*) and *BRAF*. BRAF (v-raf murine sarcoma viral oncogene homolog B1) protein is the most abundant and potent in the RAF family and is able to phosphorylate and activate MEK, which in turn phosphorylates and activates ERK (or MAPK). When activated by RAS, ERK phosphorylates and cytoplasmic proteins are translocated into the nucleus, where they regulate transcription of genes involved in cell differentiation, proliferation and survival [31, 32].

The vast majority of *BRAF* mutations found in thyroid cancer are a thymine to adenine transversion at nucleotide 1799 (T1799A) leading to a substitution of valine by glutamic acid at residue 600 of the protein (V600E). This point mutation leads to constitutive activation of BRAF kinase and chronic stimulation of the MAPK pathway, and is tumorigenic for thyroid cells. [33] This mutation is the most frequent in PTC (40–45%), also occurs in 20–40% of poorly differentiated thyroid carcinomas and 30–40% of anaplastic thyroid carcinomas [13].

Other significant mutations in *BRAF* are a substitution of lysine by glutamate in residue 601, some in-frame insertions and deletions nearly codon 600 and also the A-kinase anchor protein

9 (AKAP9)/BRAF rearrangement resulting from the fusion between the portion of the *BRAF* gene that encodes the protein kinase domain and the *AKAP9* gene[13].

1.2.3.2. *RAS* mutations

RAt Sarcoma (*RAS*) genes code for multiple G proteins that are involved in intracellular signaling through the RAF-MEK-MAPK kinase pathway. Point mutations in the *N-RAS*, *H-RAS*, and *K-RAS* genes produce continuously activated signaling proteins leading to uncontrolled growth [34].

In thyroid carcinomas the most common activating mutations are in *N-RAS* codon 61 and *H-RAS* codon 61. *RAS* mutations are found in all types of thyroid carcinomas derived from follicular cells, with different prevalence (10–20% of papillary carcinomas, 40–50% of follicular carcinomas and 20–40% of poorly differentiated and anaplastic carcinomas) [13]. Regarding the prognosis, the role of *RAS* mutations is not well established. In certain cases, *RAS* mutations were associated with aggressive tumor phenotypes and poor prognosis [35], whereas in others this association was not observed [36]. Metastatic capability has been attributed to *RAS* genes activation, since cases with *N-RAS* mutations are consistently associated with the appearance of haematogenous metastases, particularly to bone [37].

1.2.3.3. PAX8/PPAR-γ rearrangement

This rearrangement results from the fusion of part of the DNA- binding segment of paired box gene 8 (*PAX8*) and the peroxisome proliferator-activated receptor gamma (*PPAR-γ*); PAX8 is a thyroid transcription factor and PPAR-γ is a transcription factor that stimulates cell differentiation and inhibits cell growth. Initially the presence of this fusion protein was useful to distinguish PTC from FTC, as this rearrangement is found in FTC (frequency of 30-35%) and not in PTC. However, later studies reported the presence of PAX8/PPAR-γ rearrangement in cases of follicular variant of PTC, although at a lower frequency [38, 39].

1.2.3.4. TRK rearrangements

The neurotrophic receptor-tyrosine kinase (*NTRK1*) gene is located on chromosome 1q22 and encodes the receptor for nerve growth factor. *NTRK1* gene can be fused to three different partner genes situated in the same or other chromosome; these rearrangements are known as *TRK* rearrangements and are found in PTC [40].

1.2.3.5. Mutations in carcinoma dedifferentiation

It is believed that mutations in *BRAF* and *RAS* genes are an early event in progression of thyroid cancer, as these mutations are consistently found in well differentiated thyroid

carcinomas and in less differentiated thyroid carcinomas [41]. However, poorly and undifferentiated thyroid carcinomas present with high frequency of other mutations inexistent in well differentiated thyroid carcinomas, suggesting that these genetic alterations lead to tumor dedifferentiation. These are late events in the carcinogenic process and occur in the *TP53* and *CTNNB1* genes and mutation in genes that encode effectors of the PI3K–AKT signaling pathway (as *PIK3CA*, *PTEN* and *AKT1*) [13, 41]. *TP53* encodes the cell cycle regulator p53 and these mutations lead to inactivation of this tumor suppressor gene. *CTNNB1* encodes a ß-catenin that is involved in cell adhesion and Wnt signaling [13].

In conclusion, the undifferentiated thyroid carcinomas reflect not only the changes related to the type of differentiated carcinoma from which it has evolved, but also new genetic alterations associated with its loss of differentiated thyroid cell function.

1.3. *RET* gene and RET protein

The *RET* proto-oncogene was identified by Takahashi et al. in 1985, who reported, during a classical experiment, a novel gene re-arrangement with transforming activity in NIH 3T3 cells transfected with human lymphoma DNA. Therefore the nomenclature of the gene as REarranged during Transfection (*RET*) [42]. The human *RET* gene is localized on the chromosome band 10q11.2 [43], comprises 21 exons and has a size of approximately 55,000 bp [44]. The *RET* proto-oncogene encodes a transmembrane receptor of the TK family of proteins, with the same name of the gene, that is expressed in precursor cells of the neural crest and urogenital tract. RET protein is required for maturation of several cell lineages of the peripheral nervous system, kidney morphogenesis, and spermatogenesis [45].

Like every membrane receptor, RET protein (figure 3) is composed of three domains: an extracellular domain, a transmembrane domain and an intracellular domain. The extracellular domain is constituted by four cadherin-like repeats, a calcium binding site and a large cysteine-rich region, responsible to transduction of extracellular signals of proliferation, growth, differentiation, migration, survival and cell apoptosis. The intracellular domain is divided into 2 TK subdomains (TK1 and TK2), separated by 28 amino acids. TK1 and TK2 include tyrosine residues that are phosphorylated during receptor activation, and are involved in the activation of the signaling intracellular pathways [46].

Three isoforms of RET are generated by alternative splicing of the 3' region which differ in the number of amino acids in the carboxy - terminal tail, these isoforms are RET9, RET43 and RET51, consisting of 1072, 1106 and 1114 amino acids respectively [46]. The two main isoforms in vivo and best-characterized are RET9 and RET51, these isoforms are highly conserved over a broad range of species. Studies suggest that these isoforms have different

tissue-specific effects during embryogenesis. For example, mice with only the RET9 isoform are viable and normal, contrary to the monoisoformic RET51 mice that have kidney hypoplasia and lack enteric ganglia in the colon [47].

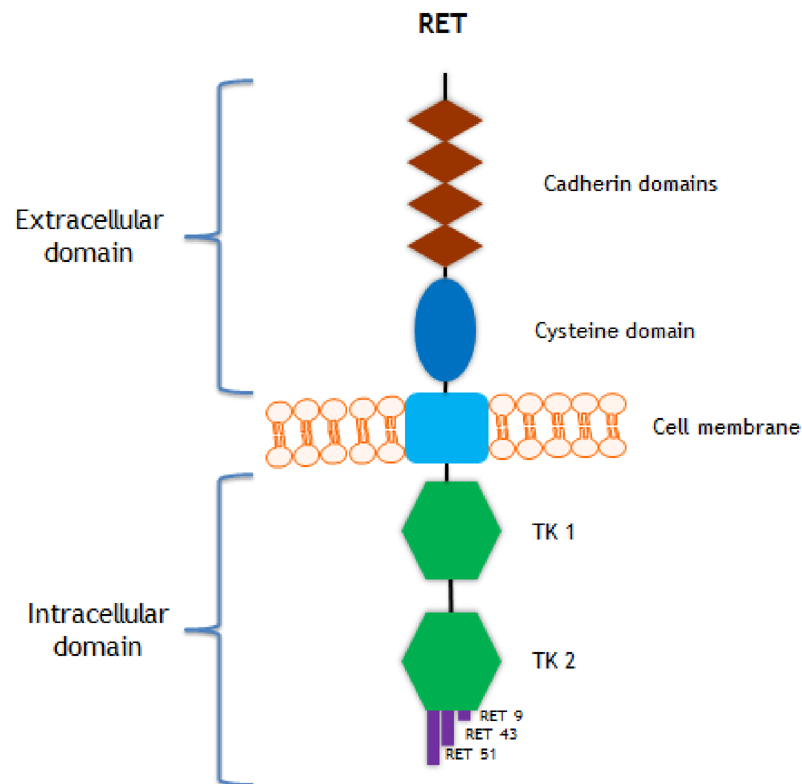

Figure 3 - Schematic representation of the RET receptor tyrosine kinase. The extracellular domain includes four cadherin domains and a cysteine rich domain. A single transmembrane region spans the cell membrane. The intracellular domain contains two tyrosine kinase domains (TK1 and TK2). The three RET isoforms (RET9, RET43 and RET51) are indicated.

The main ligands of the RET receptor TK belong to the glial cell line-derived neurotrophic factor (GDNF) family of ligands (GFLs), namely GDNF, neurturin (NRTN), persesephin (PSPN) and artemin (ARTN). The GFLs bind to and activate the RET receptor when bound to the GDNF receptor-alpha (GFRα) family.

Four different GFRα co-receptors have been characterized (GFRα1 to GFRα4), these differ in their specificity for GFLs. The primary ligands for the co-receptors GFRα1, GFRα2, GFRα3, and GFRα4 are GDNF, NRTN, ARTN, and PSPN, respectively. However, signs of cross-talk between ligands and co-receptors have been observed *in vitro* [48]. Finally the complex GFL-GFRα binds to the extracellular domain of RET and this conjugation leads to auto-phosphorylation of the intracellular tyrosine residues [49].

Usually, GFRαs are bound to the plasma membrane; however they can also occur in a soluble form (non-membrane bound). Therefore, RET can be activated in two different forms: *cis* and *trans* (figure 4). In the *cis* RET activation model the GFL binds to GFRα anchored on a lipid platform, this complex will promote the dimerization of RET, allowing the phosphorylation of the intracellular tyrosine residues. In the *trans* RET activation model the GFL binds to the soluble form of GFRα stimulating the dimerization of RET outside the lipid platform, thus allowing tyrosine residues phosphorylation [26]. Independently of the form of activation, once activated, RET begins the different intracellular pathways involving the regulation of many processes such as differentiation, survival, proliferation, migration and cell chemotaxis [26].

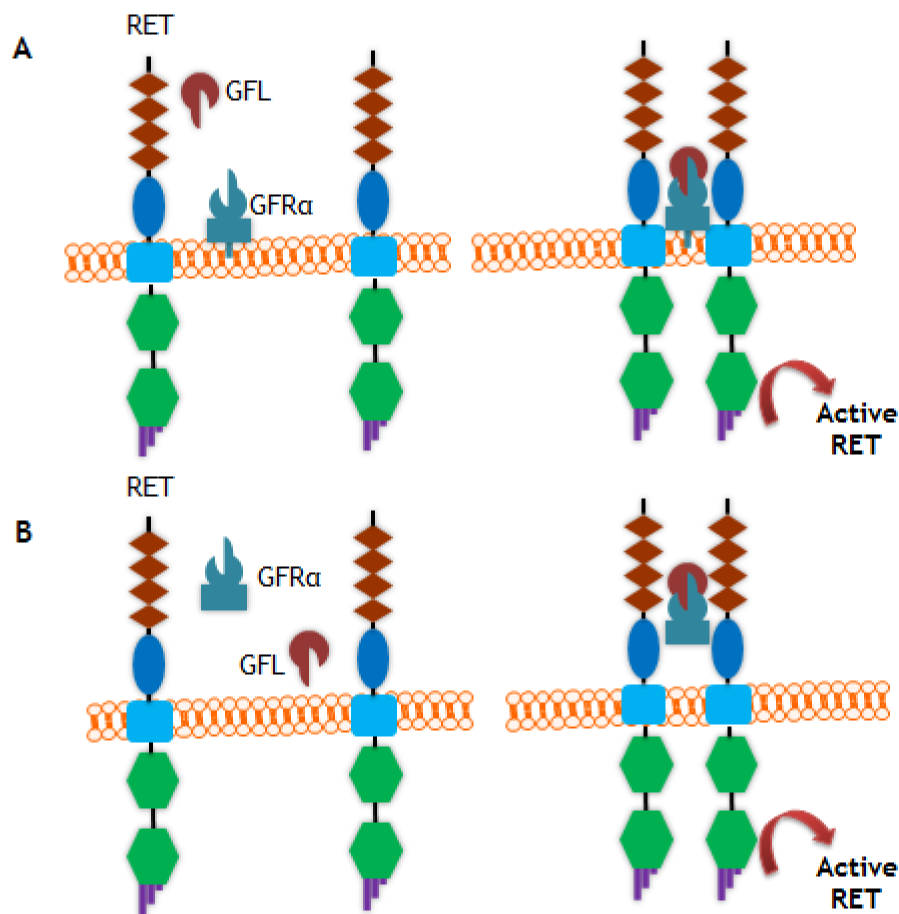

Figure 4 - Different mechanisms of ligand-mediated RET activation. A: *cis* RET activation model, the GFL binds to GFRα anchored on a lipid platform, this complex will promote the dimerization of RET and allowing the phosphorylation of the intracellular tyrosine residues. B: *trans* RET activation model, the GFL binds to the soluble form of GFRα stimulating the dimerization of RET outside the lipid platform, thus allowing tyrosine residues phosphorylation

The RET receptor is mainly activated by GFLs and GFRα co-receptors, however other growth factors can activate this receptor. Studies have proved that, through an inter-receptor-kinase-signaling-mechanism independently of ligands or co-receptors, binding of nerve growth

factor (NGF) to its receptor TK (NTRK1) modulate the phosphorylation of isoform RET51. This mechanism results in increased growth, metabolism, and gene expression [50].

As NGF activates only RET51 phosphorylation and not the phosphorylation of the other isoforms, it is improbable that NGF would simply modulate the levels of GDNF protein. These results show how growth factors and their receptors engage in cross-talk to form a dynamic network of inter-related trophic signals that guide development. The molecular mechanism of the cross-talk between NTRK1 and RET appears to be indirect, however this mechanism remains unknown [51].

1.4. Genetic alterations in *RET*

Alterations of the *RET* proto-oncogene are associated with a variety of disorders, since *RET* is a proto-oncogene, a single activating mutation in one allele is sufficient to cause neoplastic changes [52]. Loss of function mutations of the *RET* proto-oncogene results in Hirchsprung disease, a genetic disorder characterized by congenital absence of enteric neurons in the gastrointestinal tract. In contrast, gain of function mutations leading to aberrant *RET* activation, are involved in a number of human cancers such as MTC [53].

1.4.1. *RET* and follicular-cell-derived carcinomas

The clinical relevance of *RET* in human diseases was first recognized in PTC. The chromosomal rearrangement RET/PTC is found in PTC (table 2) and results by fusion of the 3' portion of *RET* with one of several possible partner genes. The *RET* proto-oncogene is expressed at very low levels in thyroid follicular cells, however when the TK domain of the RET protein is fused to genes with a promoter that is active in the follicular cells, this causes ligand-independent dimerization of the RET/PTC protein, which leads to chronic stimulation of MAPK signaling in thyroid follicular cells [54].

So far there are about 12 different fusion partner genes that vary according to the identity of the fusion partner of *RET* [55]. RET/PTC1 (60-70%) is the most common followed by RET/PTC3 (20–30%), which are fused to either *CCDC6* or *NCOA4*, respectively. These prevalent rearrangements result from paracentric inversions on chromosome 10, as fusion partners reside on chromosome 10 [8].

An association between RET/PTC and exposure to external radiation was established, as RET/PTC rearrangements have been found in over 60% of PTCs emerged from the nuclear power plant disaster in Chernobyl on 26 April 1986 [56]. Also patients previously subjected to external irradiation for benign or malignant disease showed a high prevalence of RET/PTC

rearrangements [57]. More recent studies showed frequent RET/PTC rearrangements in sporadic PTC from children and adults that were not exposed to irradiation [58]. Regarding FTC, no associations with the *RET* proto-oncogene have been established.

1.4.2. *RET* and medullary thyroid carcinomas

Germline mutations of the *RET* proto-oncogene confer predisposition to hereditary MTC (Table 1). The mutations can be divided into two main groups: (a) Mutations affecting the extracellular domain: These primarily involve cysteine residues 609, 611, 618 and 620 (exon 10), and 634 (exon 11). (b) Mutations affecting the RET-TK domain: These involve codons 768, 790 and 791 (exon 13), 804 (exon 14), 883 and 891 (exon 15), and 918 (exon 16) [39].

About 98% of MEN-2A families have germline mutations of one of the five conserved cysteine residues in exon 10 (codons 609, 611, 618, and 620) or exon 11 (codon 634) in the *RET* proto-oncogene extracellular domain, this mutations convert a cysteine into another amino acid leading to RET constitutive activation. The mutations affecting codon 634 are the most common in MEN-2A, where the substitution of the cysteine for an arginine represents 50% of all cases. Rarer mutations that are associated with MEN-2A include those at codons 768, 790 and 791 (exon 13), 804 (exon 14), and 891 (exon 15) [39, 59].

In the case of FMTC the majority of the germline mutations are in codons 618 and 620 (exon 10) and a fewer in codons 630, 631 or 634 (exon 11). Recently, mutations in other exons have been associated to FMTC, like exon 13 (codons 768, 790 and 791), exon 14 (codon 804 and 844), and exon 15 (codon 891), which are located in the TK domain, thereby interfering with intracellular ATP binding. Approximately 95% of MEN-2B families have a single mutation that converts methionine to threonine at codon 918 (exon 16), other rare intracellular mutations associated with MEN-2B are in codon 882 (exon 15) [59].

In the case of sporadic MTC, no *RET* germline mutations are found. However a significant part of these MTCs has a somatic mutation in *RET*. In these cases the mutation is only present in the tumor and the codon 918 is the most affected, but rare mutations in codons 634 and 883 have also been associated with sporadic MTC [60].

1.5. *RET* Polymorphisms and Haplotypes in human diseases

A genetic locus is considered polymorphic when one or more of the rarer alleles have a frequency of at least 1% in a population. Most polymorphisms are silent, that is, do not alter

the functional activity of the encoded protein; however there are some polymorphisms that are not neutral.

A set of closely linked genetic markers or polymorphisms that are transmitted through generations as a unit is called a haplotype. When there is correlation or association of a polymorphism or haplotype with a particular phenotype it is possible that the polymorphism or haplotype functions as a genetic modifier and is associated with a small to moderate increase in risk of developing a disease. In addition polymorphisms may also interact with other genetic variants modulating their action. Another important point for the study of polymorphisms is that these are relatively common, so that they can present a much greater risk in a population than rare mutations in genes with high susceptibility to cancer as the *RET* proto-oncogene [61].

The polymorphisms are divided into: minisatellite and microsatellite sequence polymorphisms and single-nucleotide polymorphisms (SNPs). SNPs represent approximately 90% of all the human genome variations and occur every 100 to 300 bases along the three-billion-base human genome and are the most commonly used genetic markers. A SNP which leads to modification of the amino acid sequence of the protein that is produced is called nonsynonymous SNP. When the SNP does not lead to a change in amino acid sequence, it is designed synonymous SNPs [62]. There are about 11 million SNPs described in the human genome, which are distributed uniformly and, so, these are important biomarkers in screening of complex diseases [63].

Carcinogenesis is a multistep process that occurs through an interaction between many genetic and environmental factors. Thus it is unlikely that the effect of a single polymorphism in this process is substantial. However, it has been shown that an approach based on the combined polymorphisms that interact in the same way can amplify the effect of individual variants and improve the predictive power of polymorphism analysis of complex diseases [64].

Over the last years, many groups have used SNPs of the *RET* proto-oncogene to determine whether polymorphic variants might represent low penetrance alleles predisposing to *RET* associated disorders. Several SNPs of this gene have been described in the general population as well as in patients with endocrine tumors, PTC, familial and sporadic MTC and Hirchsprung disease. An issue still unclear is how polymorphisms could have interacting, predisposing, or modifying roles in the pathogenesis of these diseases [65].

1.5.1. *RET* Polymorphisms and Haplotypes in MTC

Large improvements have been made in the study of the pathogenesis of MTC since the *RET* proto-oncogene was identified as the susceptibility gene for MTC. But there are many aspects about the MTC, principally about the sporadic form, that remain poorly understood. For

example, the heterogeneity at clinical level observed in individuals with the same mutation [26].

In the last few years various studies have been made to understand if the presence of certain *RET* polymorphisms are associated or not with the susceptibility for the development or progression of MTC. In general 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 to the general population.

Nevertheless these results are still controversial, as several studies indicate an involvement of *RET* polymorphisms in the development of sporadic MTC, whereas several others failed to demonstrate an association between these *RET* polymorphisms and MTC development or progression [26].

1.5.1.1. *RET* G691S and S904S Polymorphisms

The *RET* polymorphism G691S (Gly/GGT → Ser/AGT) is a non-synonymous variant present in exon 11 which leads to the alteration of a glycine to a serine in the protein. The *RET* polymorphism S904S (Ser/TCC → Ser/TCG) is a synonymous variant located in exon 15.

Two large studies, one in an Italian population and the other in a British population, demonstrated that the *RET* variant G691S is more frequent in MTC patients than in the general population. These two studies hypothesized, through a functional assessment of *RET* transcription and splicing, that G691S could be the functional variant, but the results were inconclusive [66, 67]. Additionally the Elisei et al. (2004) study described a positive significant co-segregation between G691S and S904S Polymorphisms [66].

The co-segregation between these two polymorphisms in MTC cases were confirmed in a Spanish population by Robledo et al. (2003), reporting a strong linkage disequilibrium. Other conclusions of this study were the higher prevalence of the haplotype G691S/S904S, in homozygosity, in patients with MEN 2A compared to the control group; the authors also observed that this haplotype could modify the age of onset of MTC patients [68]. In the Portuguese population, one study described an over-representation of the G691S polymorphism, particularly in females, in MTC patients with respect to controls, although not reaching the level of significance [69].

Although various authors have demonstrated an association between G691S/S904S polymorphisms and MTC, some other studies did not find a difference in the frequency of these polymorphisms between MTC patients and the general population. These negative results were found and replicated in studies with Polish, Indian and Brazilian populations [62, 70, 71].

1.5.1.2. *RET* L769L Polymorphism

The *RET* L769L polymorphism (Leu/CTT → Leu/CTG) is a synonymous variant present in exon 13. A study developed by Wiench et al. (2001) described that patients with MTC younger than 30 years presented a higher frequency of the *RET* L769L variant than those diagnosed between 31 and 45 years, but the absence of a control group decreased the relevance of this finding [72].

Baumgartner-Parzer et al. (2005) described an association between the presence of L769L polymorphism and F791Y mutation in FMTC patients. the authors deduced that the F791Y mutation and L769L polymorphism are located on the same allele and predicted 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 [65].

The *RET* L769L polymorphism was also associated with an increased risk for MTC whereas patients homozygous for the minor allele of this variant were younger at the MTC diagnosis [62]. On the other hand, several studies in distinct populations failed to demonstrate an association between this polymorphism and MTC [70, 71, 73].

1.5.1.3. *RET* S836S Polymorphism

The *RET* S836S polymorphism is a neutral variant and is located in exon 14. Gim et al. (1999) developed a study that reported a higher frequency of this variant minor allele in MTC patients comparing to a control group, Ruiz et al. (2001) obtained similar data in Spanish population [74, 75]. Another interesting finding on Gim et al. (1999) study was that eight of nine patients (89%) with the minor allele of the S836S polymorphism also had a somatic mutation in the M918T [75]. A large study in a Brazilian population associated the variant S836S with early onset of sporadic and hereditary MTC and also with the formation of lymph nodes and distant metastases [70].

In spite of this, other association studies failed to demonstrate any differences in the presence of this polymorphic allele between MTC patients and controls. For example the studies in Polish, French and Indian populations demonstrated a similar frequency in the MTC patients and in the controls groups [62, 71, 73].

1.5.2. *RET* Polymorphisms and Haplotypes in DTC

So far, only a few studies have evaluated the association between *RET* SNPs and DTC. As in MTC, in DTC, polymorphic variations in the DNA sequence of *RET* might modify expression of the RET protein and/or lead to activation of the gene, so it is expected that much still remains to be discovered [76].

Lesueur et al. (2002) conducted an association study with PTC cases and controls from four countries (France, Portugal, Italy and Australia) matched for sex, age, and population. The four *RET* SNPs studied were A45A in exon 2, L769L in exon 13, S836S in exon 14, and S904S in exon 15 and, also, a total of 10 haplotypes. The authors concluded that some *RET* polymorphic variants and some specific haplotypes could have low penetrant alleles for the PTC phenotype. The strongest association with PTC was found for A45A and L769L polymorphisms, moreover a specific haplotype, designed GGCC haplotype, might act as a low penetrance predisposing allele for PTC in the Italian and French populations [76].

Other studies demonstrated a weak association with PTC and the SNPs A432A, S836S, G691S, and S904S. Ho et al. (2005) described an association with DTC for A432A and S836S polymorphisms [14]. Stephens et al. (2005) investigated loss of heterozygosity (LOH) for three *RET* SNPs (G691S, S904S, and L769L) in patients from Ukraine and Belarus that developed PTC due to exposition to radioactive fallout following the Chernobyl nuclear accident and in a control group. The results showed an association with PTC for G691S and S904S [77].

Taking into account all these studies, the dimension of the effect among *RET* SNPs and/or haplotypes and PTC is only slightly significant. This association should be confirmed on larger samples.

1.6. Aims of the present study

Polymorphisms of the *RET* proto-oncogene have been studied in different populations for association with susceptibility to thyroid cancer, but with inconsistent findings mainly in thyroid carcinomas derived from follicular cells. There are multiple approaches to identify susceptibility genes and biological mechanisms in a complex disease. Association studies, one of the most used methods, seek to identify genetic variants that are associated with pathology, comparing affected individuals with a control population.

To clarify the contribution of *RET* polymorphisms to genetic susceptibility to DTC among Portuguese patients, we conducted an association case-control study by analyzing four well-characterized *RET* polymorphisms (G691S, L769L, S836S and S904S). This general goal can be divided into three points: i) to determine the prevalence of these *RET* polymorphisms in the Portuguese population; ii) to determine the association of these *RET* polymorphisms and haplotypes with DTC; iii) to define the association of these *RET* polymorphisms and haplotypes with clinical parameters (cancer subtype, gender, age at diagnosis and tumor size at diagnosis).

2. Materials and methods

2.1. Studied population

The study group consisted of 282 Caucasian Portuguese patients with thyroid cancer (47 males and 234 females; mean age ± SD, 48.5 ± 14.8 years, median 49) from the *Instituto Português de Oncologia de Coimbra*. The control group consisted of 245 (100 males and 145 females; mean age ± SD, 32.0 ± 14.3 years, median 25) Caucasian Portuguese unrelated volunteers who were recruited among faculty staff or blood donors of the *Instituto Português de Sangue da Região Centro*, with no history of thyroid cancer. This study was approved by local research ethics committee.

2.2. Genotyping

2.2.1. DNA Extraction

A total of 10 mL of venous blood was collected, in EDTA tubes, from each individual. Genomic DNA was extracted from peripheral blood leucocytes collected and stored at 4ºC until manipulation. The DNA extraction procedure was based on the "salting-out" method, described by Miller [78].

The whole blood sample was transferred to a 50mL falcon tube and the final volume was completed until 45mL with Red Blood Cell (RBC) lysis buffer (155 mM NH4Cl; 20 mM KHCO3; 0.1 mM Na2EDTA; pH 7.4). The tubes were vortexed and incubated on ice for 15 minutes, with frequent inversions. After this incubation period, the tubes were centrifugated (AllegraTM X-22R Centrifuge, Beckman Coulter, USA) at 2500 rpm for 10 minutes at 4ºC, then, the supernatant was discarded, leaving the leucocytes intact.

Once the leucocytes were isolated, the pellet was completely resuspended and incubated with 5mL of Secundary Extraction buffer (75 mM NaCl; 25 mM Na2EDTA; pH 8.0), 12.5 µL proteinase K (20 mg/ml) and 500 µL SDS 10%. The digestion was left overnight at 55ºC. To precipitate the proteins 3 mL of saturated NaCl (6M) were added followed by a 10 minutes incubation at 55ºC, tubes were vortexed and centrifuged at 4000 rpm for 30 minutes at 15ºC.

The supernatant was decanted to a new 50 mL falcon tube, avoiding foam. To perform the DNA precipitation, cold 100% ethanol was added at about 2x the volume of the supernatant, tubes were inverted gently about 50 times observing the formation of the DNA precipitate and centrifuged at 4500 rpm for 5 minutes at 4ºC, the supernatant was decanted and discarded. The DNA pellet was washed with 10 ml of cold 70% ethanol and centrifuged at 4500 rpm for 5

minutes at 4ºC, finally was transferred into a 1.5 mL microfuge tube and allow to air dry for about 30 minutes. DNA was hydrated with 1mL of Tris-EDTA buffer and left in slow and constant stirring (Programmable Rotator Mixer, Star Lab) overnight at room temperature.

When the blood sample was insufficient to implement the protocol described above, the DNA was extracted using the Puregene Blood Core Kit C (Qiagen), according to the manufacturer's instructions.

2.2.2. DNA quantification

Accurate analysis of the DNA preparation may be impeded by the presence of impurities in the sample or if the amount of DNA is too little or too high, so it is necessary to quantify the samples.

DNA quantity and quality was assessed by spectrophotometric determination (Nanophotometer™, Implen, Germany). Analysis of UV absorption by the nucleotides provides a simple and accurate estimation of the concentration of nucleic acids in a sample. Purines and pyrmidines in nucleic acid show absorption maxima around 260nm (dATP: 259nm; dCTP: 272nm; dTTP: 247nm) if the DNA sample is pure without significant contamination from proteins or other components. To assess the purity of the samples, the ratio of OD_{260}/OD_{280} was determined. A ratio between 1.8-2.0 denotes that the absorption in the UV range is due to nucleic acids. A ratio lower than 1.8 indicates the presence of proteins and/or other UV absorbers. A ratio higher than 2.0 indicates that the samples may be contaminated by RNA. After DNA quantification all samples were diluted to the same concentration (100 ng/µL).

2.2.3. Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) was developed by Kary Mullis in 1985 and is used to exponentially amplify a specific DNA fragment, *in vitro* [79].

The amplification of *RET* exons 11, 13, 14 and 15 was conducted using specific primers and optimized PCR conditions (see table 2). The PCR reactions of *RET* exons 11, 13 and 14 occurred in a final volume of 25 µL: 1X buffer (20 mM Tris HCL; pH 7.5; 100 mM NaCl; 0.1 mM EDTA; 1mM dithiothreitol; 50% (v/v) glycerol), 0.2 mM of dNTPs, 0.2 µM of each primer (forward and reverse), optimized concentration of $MgCl_2$ (Table 2), 1 U of NZYTaq (NZYtech Lda., Lisbon, Portugal) and 100-300 ng of genomic DNA. The amplification of exon 15 was performed using Supreme NZYTaq 2X Green Master Mix with 2,5 U of Taq (NZYtech Lda., Lisbon, Portugal), optimized concentration of $MgCl_2$ (table 2) and 100–300 ng of genomic DNA. The PCR reaction occurred in a thermal cycler (T100TM Thermal Cycler, Bio Rad) and the amplification thermal cycling profile was: initial denaturation at 95ºC for 5 minutes, 35 cycles of denaturation of 30 sec at 95ºC, annealing for 30 sec at a particular temperature dependent

on the primer sequences (Table 2), and an extension for 30 sec at 72ºC. This was followed by a final extension step of 7 minutes at 72ºC. Negative and positive controls were included in each amplification analysis.

Table 2 - Primers and PCR conditions for amplification of gene *RET* exons 11, 13, 14 and 15.

Exon	Primers (5'-3')	$MgCl_2$ (mM)	Annealing temperature (ºC)	Fragment (bp)
11	F: GGTCTAGGAGGGGGCAGTAAATGG R: CAGCGTTGGCAGCCCCTCACAG	1.5	63	562
13	F: AGAAGCCTCAAGCAGCATCGTC R: AGGAGCAGTAGGGAAAGGGAGAAA	2.0	63	348
14	F: CACGAGCAGCAGGAGGCAGAGA R: GAGTGTGGCATGGTGGGGGAGTGG	1.0	63	549
15	F: CCCCCGGCCCAGGTCTC R: GCTCCACTAATCTTCGGTATCTTT	2.0	65	358

2.2.4. Enzymatic digestion

The amplified PCR products were subjected to enzymatic digestion because all the polymorphisms create or abolish a restriction site for a specific endonuclease. The *BanI*, *TaqI*, *AluI*, and *RsaI* enzymes (New England Biolabs, Beverly, MA, USA) were used to study the G691S, L769L, S836S, and S904S polymorphisms, respectively.

BanI was used to distinguish the *RET* exon 11 G691S polymorphism in which the homozygous common GG genotype produced 4 fragments (17, 147, 175 and 223, bp). The homozygous AA genotype abolish one *BanI* restriction site, so 3 fragments (17, 147, 398 bp) were produced and the heterozygous GA genotype resulted in 5 fragments (17, 147, 175, 223, 398 bp) (table 3). Digestion of 10 µL of PCR product was performed in 15 µL reaction volume using 5 units of *BanI*, 1X NEbuffer 4 (50 mM potassium acetate; 20 mM Tris-acetate; 10 mM magnesium acetate; 1 mM dithiothreitol; pH 7.9) and 100 µg/mL of BSA supplied with the restriction enzyme at 37ºC overnight.

TaqI enzyme was used in order to identify the *RET* exon 13 L769L polymorphism. The homozygous common TT genotype produced 2 fragments (164, 184 bp). The homozygous GG genotype abolishes the *TaqI* restriction site avoiding the digestion, remaining the fragment intact. Finally, the heterozygous TG genotype produced 3 fragments (164,184, and 348 bp) (table 3). Digestion of 10 µL PCR product was performed in 15 µL reaction volume using 5 units of *TaqI*, 1X NEbuffer 4 (50 mM potassium acetate; 20 mM Tris-acetate; 10 mM magnesium acetate; 1 mM dithiothreitol; pH 7.9) and 100 µg/mL of BSA supplied with the restriction enzyme, at 65ºC overnight.

*Alu*I was used to distinguish the *RET* exon 14 S836S polymorphism in which the homozygous common CC genotype resulted in 5 fragments (32, 42, 107, 158, 210 bp). The homozygous TT genotype resulted in loss of one *AluI* restriction site, giving rise to 4 fragments (32, 42, 107, 368 bp) and the heterozygous CT genotype produced 6 fragments (32, 42, 107, 158, 210, 368 bp) (table 3). Digestion of 10 µL PCR product was performed in a final volume of 50 µL using 5 units of *Alu*I and the 1X NEbuffer 4 (50 mM potassium acetate; 20 mM Tris-acetate; 10 mM magnesium acetate; 1 mM dithiothreitol; pH 7.9) supplied with the restriction enzyme, at 37ºC for an overnight period of incubation.

*The Rsa*I endonuclease was used to distinguish the *RET* exon 15 S904S polymorphism in which the homozygous common CC genotype resulted in the absence of *RsaI* restriction sites, thus the fragment (358 bp) remained intact. The homozygous GG genotype resulted in 2 fragments (123, 235, bp). And the heterozygous CG genotype produced 3 fragments (123, 235, 358 bp) (table 3). Digestion of 10 µL PCR product was performed in 50 µL reaction volume using 5 units of *Rsa*I and the 1X NEbuffer 4 (50 mM potassium acetate; 20 mM Tris-acetate; 10 mM magnesium acetate; 1 mM dithiothreitol; pH 7.9) supplied with the restriction enzyme at 37ºC overnight.

Table 3 - Gene *RET* polymorphisms, expected genotypes and the respective restriction fragments.

Polymorphism	Genotype	Fragments (bp)
	GG	17, 147,175, 223
G691S	AA	17, 147, 398
	GA	17, 147, 175, 223, 398
	TT	164, 184
L769L	GG	348
	TG	164, 184, 348
	CC	32 42, 107, 158, 210
S836S	TT	32, 42, 107, 368
	CT	32, 42, 107,158, 210, 368
	CC	358
S904S	GG	123, 235
	CG	123, 235, 358

2.2.5. Electrophoresis

In order to confirm the PCR amplifications and the enzymatic digestions, electrophoretic migration was performed using an agarose polymer (Seakem® LE Agarose, Lonza). The

agarose gel was prepared by boiling agarose in 1X TAE buffer and stained with ethidium bromide (1 µL/mL). For PCR products the gel has 1.5% (m/v) of agarose and for enzymatic digestion products the gel has 3.0% (m/v) of agarose. Then, 5 µL of the final products was loaded. A molecular weight marker (HyperLadder II, Bioline or VC 100 bp Plus DNA Ladder, Vivantis) was used as a size standard.

After loading, the gels ran at specific voltage conditions (1.5% (m/v) agarose gel, at 145 V for about 25 minutes and the 3.0% (m/v) agarose gel was run at 130V for about 45 minutes). Finally, the fragments were visualized under UV light using UVITEC system (Uvitec Cambridge).

2.2.6. DNA sequencing

Confirmation of genotyping results were performed by DNA sequencing of samples, representing the three possible genotypes for each studied polymorphism.

Before sequencing the PCR products had to be purified, in order to eliminate or neutralize PCR residuals, this purification was performed by enzymatic purification. To a PCR product of 10 µL 1 unit of *FastAP* enzyme and 10 units of *ExoI* enzyme were added (Thermo Scientific). The mixure was then submitted to two incubation periods one of 20 minutes at 37ºC followed by another of 20 minutes at 80ºC.

DNA sequencing was carried out following the protocol GenomeLab™ Dye Terminator Cycle Sequencing with Quick Start Kit for GenomeLab™ GeXP sequencer, Genetic Analysis System from Beckman Coulter. Sequence data was analyzed using GenomeLab system Beckman Coulter version 10.2 software.

2.3. Statistical analysis

Statistical analysis was carried out in order to evaluate the association between the four polymorphisms in this study and development of thyroid cancer.

The allelic and genotypic frequencies for each polymorphism in patients and controls were determined by direct counting. For each polymorphism, deviation of the genotype frequencies from those expected under Hardy-Weinberg equilibrium (HWE) was assessed in controls. The evaluation of the HWE was performed by calculating the expected genotype frequencies and comparing them with the observed frequencies. HWE is based on the following principal assumptions: (i) mating is random; (ii) allelic frequencies are conserved from generation to generation; (iii) no significant migrations occur; (iv) mutation, selection,

genetic drift and gene flow are negligible [80]. All of these requirements were assumed to be true while calculating the expected genotype frequencies. The agreement between observed and expected values was tested by the chi-square (X^2) test. The linkage disequilibrium (LD) coefficients, D' and r^2, were assessed by Haploview version 4.1 (they range from: $-1 < D'<10$ and $< r^2 < 1$).

RET haplotypes derived from G691S, L769L, S836S and S904S polymorphisms were constructed using combinations of genotypes (e.g., an individual genotyped as CT/AA/GG/TT was considered to have haplotypes C/A/G/T + T/A/G/T). Individuals heterozygous for more than one polymorphism were not considered for haplotype frequency analysis, since the distribution of the alleles between the two homologous chromosomes is not clearly defined (e.g., a subject genotyped as CT/AG/GG/TT could have either haplotypes C/A/G/T + T/G/G/T or C/G/G/T + T/A/G/T).

Subgroup analysis was carried out to assess the effect of *RET* polymorphisms in cancer subtype (PTC and FTC), gender (male and female), age at diagnosis (cutoff at 45 years) and tumor size at diagnosis (cutoff at 10 mm of diameter). The Bonferroni's correction was used for adjustment for these multiple comparisons.

Genotype, allele and haplotype frequencies among groups were compared by Pearson's X^2-test of independence, statistical significance was taken as $p < 0.05$, when expected values were less than five, Fisher's exact test was used instead. The genotypic and allelic-specific risks were estimated as odds ratios (ORs), with associated 95% confidence intervals (CI).

3. Results

3.1. Genotyping

3.1.1. *RET* G691S polymorphism

After the fragment of 562 bp, corresponding to exon 11, was amplified by PCR from genomic DNA samples, we proceeded to view the result by electrophoresis (figure 5 (A)). The identification of G691S genotype for each individual was performed by electrophoresis after enzymatic digestion with the specific restriction enzyme (figure 5 (B)). One sample representing each genotype was sequenced to confirm the results obtained by enzymatic digestion (figure 5 (C)).

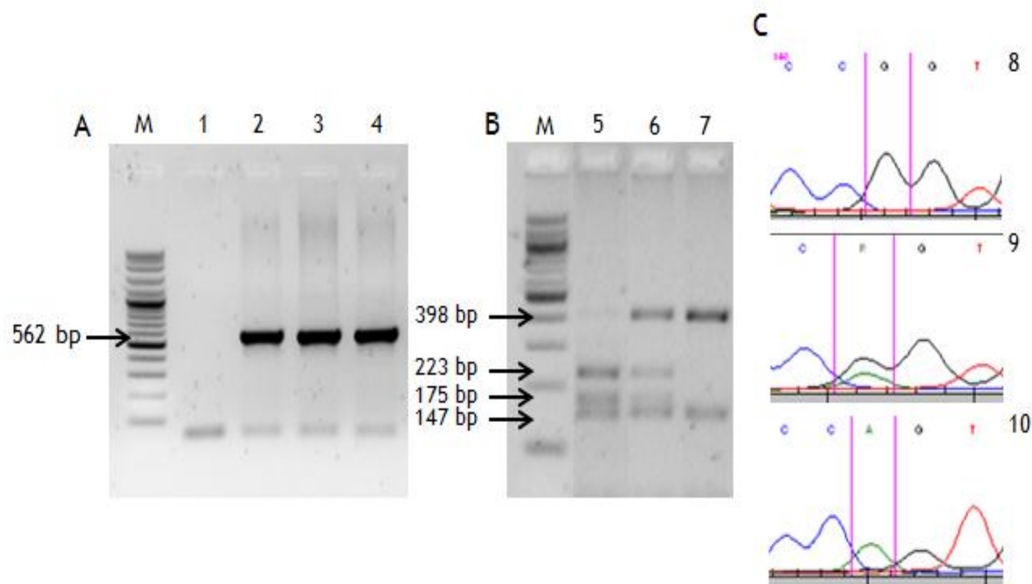

Figure 5 – Genotyping results for the G691S polymorphism. A: 1% Agarose gel electrophoresis of PCR products containing exon 11; 1 – negative control, 2 to 4 bands of 562 bp representing exon 11 PCR amplification. B: 3% Agarose gel electrophoresis representing the three genotypes of the G691S polymorphism obtained by enzymatic digestion with *BanI*, 5 - homozygous common GG genotype represented by 3 bands in the gel (147, 175 and 223 bp), 6- heterozygous GA genotype resulted in 4 bands in the gel (147, 175, 223 and 398 bp), 7- homozygous polymorphic AA genotype characterized by 2 bands in the gel (147 and 398 bp). C: Chromatograms confirming the three genotypes for the G691S polymorphism; 8 - homozygous common GG genotype, 9 - heterozygous GA genotype, 10 - homozygous AA genotype. M – molecular weight marker.

3.1.2. *RET* L769L polymorphism

Exon 13, where the L769L polymorphism is located, was amplified and the resultant products were ran in an electrophoresis to view the 348 bp fragment, as shown in figure 6 (A). In order to identify the L769L polymorphism for each individual, enzymatic digestion with *TaqI* was performed and another electrophoresis was ran to see the resultant fragments (figure 6 (B)). With the purpose of confirming that the enzymatic digestion allows the distinction of the various genotypes, 3 samples representative of the 3 genotypes for the L769L polymorphism were sequenced and the correspondent chromatograms are shown in figure 6 (C).

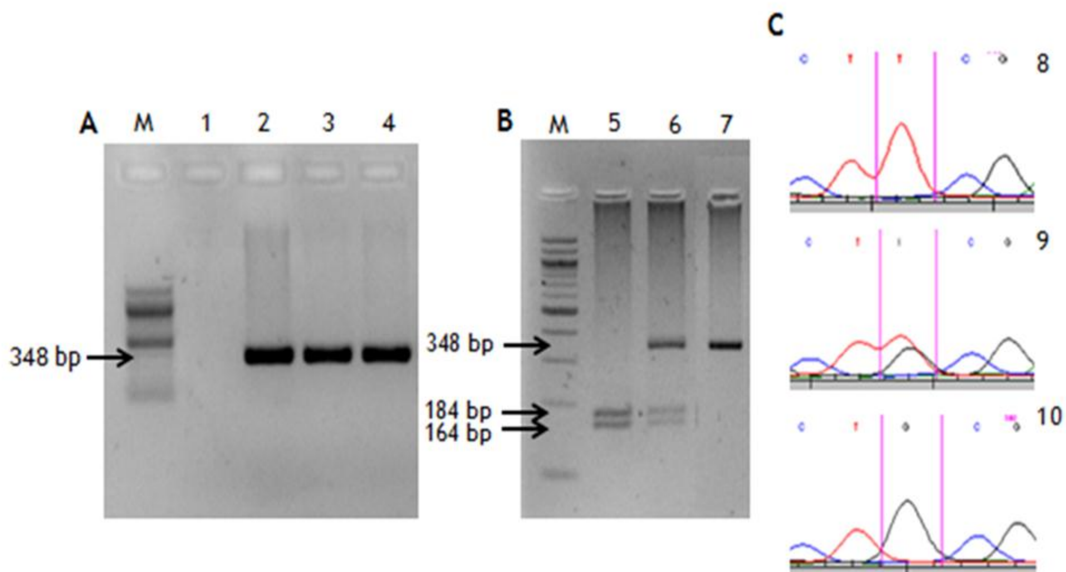

Figure 6 - Genotyping results for the L769L polymorphism. **A:** 1 % Agarose gel electrophoresis of PCR products with exon 13; 1 – negative control, 2 to 4 - bands of 348 bp representing exon 13 PCR amplification. **B: 3 %** Agarose gel electrophoresis showing the three genotypes of the L769L polymorphism obtained by enzymatic digestion with *TaqI*, 5 - homozygous common TT genotype represented by 2 bands in the gel (164 and 184 bp), 6 - heterozygous TG genotype represented in the gel by 3 bands (164, 184 and 348 bp), 7- homozygous GG genotype showed by the intact band of 348 bp. **C:** Chromatograms confirming the tree genotypes for the L769L polymorphism; 8 - homozygous common TT genotype, 9 - heterozygous TG genotype, 10 - homozygous GG genotype. M – molecular weight marker.

3.1.3. *RET* S836S polymorphism

To genotype the individuals for the S836S polymorphism, exon 14 which contains this polymorphism was amplified. The confirmation of this amplification was assessed by viewing a band of 549 bp in an agarose gel electrophoresis (figure 7 (A)). The enzymatic digestion with *AluI* resulted in the possibility of identifying the genotype of each individual for this polymorphism, as shown in figure 7 (B). To confirm these results, the sequencing of 3 individuals samples representing the 3 genotypes was performed (figure 7 (C)).

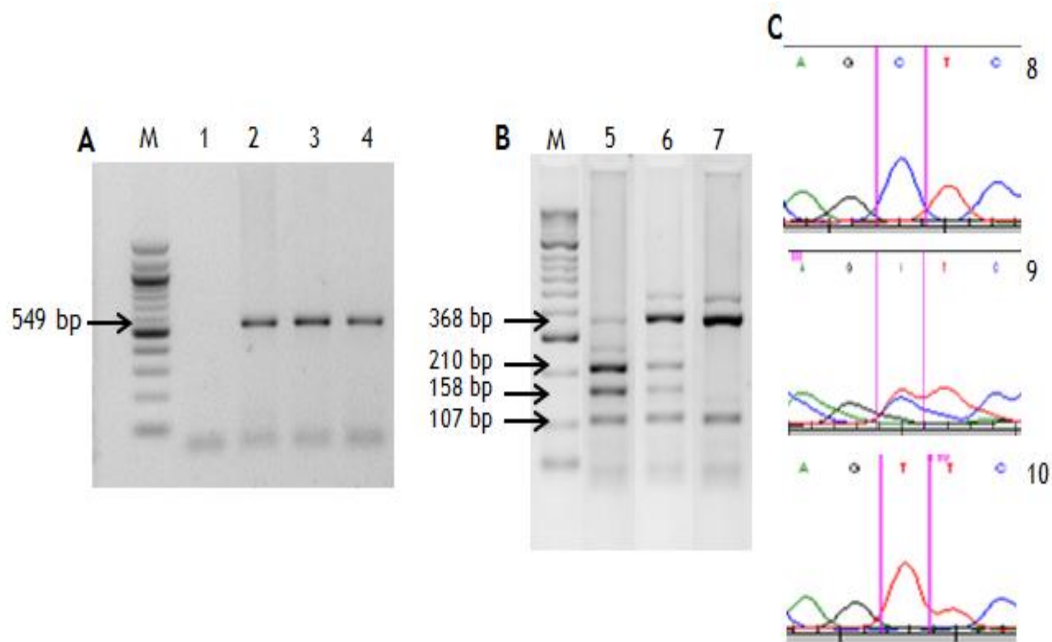

Figure 7 - Genotyping results for the S836S polymorphism. A: 1% agarose gel electrophoresis of PCR products with exon 14; 1 – negative control, 2 to 4 - bands of 549 bp representing exon 14 PCR amplification. B: 3% agarose gel electrophoresis showing the three genotypes of S836S polymorphism obtained by enzymatic digestion with AluI; 5 - homozygous common CC genotype corresponding to 3 bands in the gel (107, 158 and 210 bp), 6 - heterozygous CT genotype represented in the gel by 4 bands (107, 158, 210 and 368 bp), 7- homozygous GG characterized by 2 bands in the gel (107 and 368 bp). C: Chromatograms confirming the three genotypes for the S836S polymorphism; 8 - homozygous common CC genotype, 9 - heterozygous CT genotype, 10 - homozygous TT genotype. M – molecular weight marker.

3.1.4. *RET* S904S polymorphism

The genotyping of the S904S polymorphism was assessed by amplification of exon 15 (figure 8 (A)), followed by enzymatic digestion with *RsaI* figure 8 (B)). Three samples, representatives of the 3 genotypes for this polymorphism were sequenced, in order to confirm the results previously obtained and the corresponding chromatograms are shown in figure 8 (C).

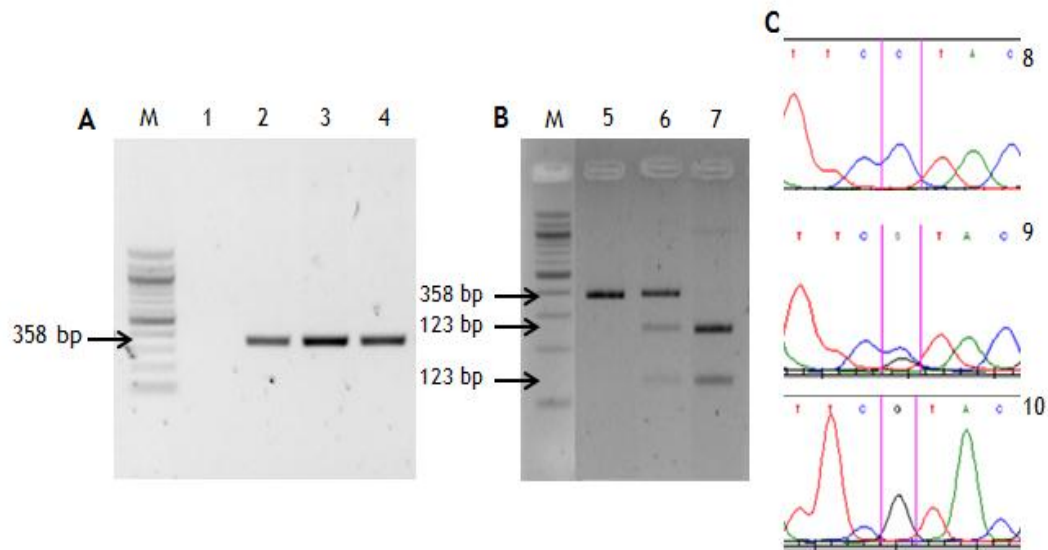

Figure 8 – Genotyping results for the S904S polymorphism. A: 1% agarose gel electrophoresis of PCR products containing exon 11; 1 – negative control, 2 to 4 - bands of 562 bp representing exon 11 PCR amplification. B: 3% agarose gel electrophoresis representing the three genotypes of the G691S polymorphism obtained by enzymatic digestion with BanI, 5 - homozygous common GG genotype represented by 3 bands in the gel (147, 175 and 223 bp), 6- heterozygous GA genotype resulted in 4 bands in the gel (147, 175, 223 and 398 bp), 7- homozygous AA genotype characterized by 2 bands in the gel (147 and 398 bp). C: Chromatograms confirming the three genotypes for G691S polymorphism; 8 - homozygous common GG genotype, 9 - heterozygous GA genotype, 10 - homozygous AA genotype. M – molecular weight marker.

3.2. Statistical analysis

3.2.1. Prevalence of the *RET* polymorphisms in a Portuguese population

No significant deviation (p>0.05) from HWE was observed for the genotype distributions for the four studied *RET* polymorphisms. In order to assess whether this Portuguese population resembles the European population, regarding the prevalence of *RET* polymorphisms, a X^2-square test was performed for each polymorphism, comparing the genotype and allele distributions between this Portuguese population and a European population constituted by 379 individuals and available in *Ensembl* database (http://www.ensembl.org/); data shown in table 4.

Table 4- Comparison of the prevalence of *RET* polymorphisms between the studied Portuguese population and a European population.

Polymorphism	This study, n(%)	European population*, n (%)	P – value**
	N = 245	N = 379	
G691S genotype			
GG	164 (66.9)	245 (64.6)	
GA	74 (30.2)	117 (30.9)	0.558
AA	7 (2.9)	17 (4.5)	
G691S allele			
G	402 (82.0)	607 (80.1)	0.390
A	88 (18.0)	151 (19.9)	
L769L genotype			
TT	158 (64.5)	218 (57.5)	
TG	81(33.1)	139 (36.7)	0.064
GG	6 (2.4)	22 (5.8)	
L769L allele			
T	397 (81.0)	575 (75.9)	0.032
G	93 (19.0)	183 (24.1)	
S836S genotype			
CC	228 (93.1)	338 (89.2)	0.103
CT	17 (6.9)	41 (10.8)	
S836S allele			
C	473 (96.5)	717 (94.6)	0.112
T	17 (3.5)	41 (5.4)	
S904S genotype			
CC	164 (66.9)	245 (64.6)	
CG	74 (30.2)	115 (30.3)	0.411
GG	7 (2.9)	19 (5.0)	
S904S allele			
C	402 (82.0)	605 (79.8)	0.785
G	88 (18.0)	138 (20.2)	

* Data available in *Ensembl* database (http://www.ensembl.org/)
**Data compared using X^2 test

P-values were greater than 0.05 in all cases with the exception of the L769L polymorphism (p=0.032). As a result, for a confidence interval of 95%, there are no differences in the prevalence of G691S, S836S and S904S *RET* polymorphisms between the studied Portuguese population studied and a European population. The distribution of L769L *RET* polymorphism in this Portuguese population differs from a previously studied European population.

3.2.2. Linkage disequilibrium coefficients between the *RET* polymorphisms in a Portuguese population

D' and r^2 are the two most important measures for two-locus haplotype analysis, since LD is non-quantitative there is no natural scale for measuring it [81]. The pairwise linkage disequilibrium coefficients D’ and r^2 in the studied Portuguese population are given in table 5, and Figure 9 is the LD plot correspondent to the indicated r^2.

Table 5 - Pairwise linkage disequilibrium coefficients (D' and r^2) between the *RET* polymorphisms in the control group.

Polymorphism	G691S		L769L		S8363S		S904S	
	D'	r^2	D'	r^2	D'	r^2	D'	r^2
G691S	-	-	-1.00	0.05	-1.00	0.01	0.94	0.89
L769L	-	-	-	-	0.64	0.06	-1.00	0.05
S836S	-	-	-	-	-	-	-1.00	0.01
S904S	-	-	-	-	-	-	-	-

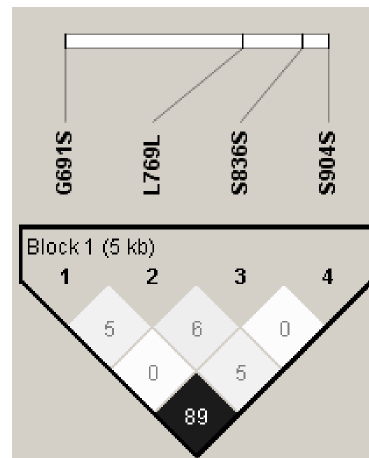

Figure 9 - Linkage disequilibrium (LD) plot of *RET* polymorphisms. The LD plot was constructed by Haploview version 4.1. The shade of squares illustrates the strength of pairwise r^2 values on a black and white scale. Black indicates perfect LD (r^2 = 1.00) and white indicates perfect equilibrium (r^2 = 0), shaded gray indicates 0 < r^2 < 1. The r^2 LD value is also indicated within each square.

Observing these findings, the higher LD is between the G691S and S904S polymorphisms (D'= 0.94; r^2 = 0.89), followed by the LD between the L769L and S836S polymorphisms (D' = 0.64; r^2 = 0.06).

3.2.3. Analysis of *RET* polymorphisms and haplotypes in thyroid cancer

To understand the role that *RET* polymorphisms may have in the development of follicular cell-derived thyroid carcinomas, we first investigated the genotype, allele and haplotye distributions of G691S, L769L, S804S and S904S SNPs in patients with PTC and FTC pooled together. The genotype, allele and haplotype frequencies observed in patients were compared with the correspondent frequencies in the control group (data shown in table 6). Odds ratio provided for each haplotype corresponds to the comparison of the haplotype with all the others.

Table 6 - Single locus analysis of *RET* polymorphisms and respective haplotypes between patients and controls.

Polymorphism	Controls, n (%) N = 245	Patients, n (%) N= 282	OR	95% CI	p-value
G691S genotype					
GG	164 (66.9)	182 (64.5)	0.90	(0.63 - 1.29)	0.563
GA	74 (30.2)	87 (30.9)	1.03	(0.71 - 1.50)	0.872
AA	7 (2.9)	13 (4.6)	1.64	(0.64 - 4.19)	0.294
G691S allele					
G	402 (82.0)	451 (80.0)	0.87	(0.64 - 1.19)	0.392
A	88 (18.0)	113 (20.0)	1.14	(0.84 - 1.56)	
L769L genotype					
TT	158 (64.5)	179 (63.5)	0.96	(0.67 - 1.37)	0.809
TG	81(33.1)	88 (31.2)	0.92	(0.64 - 1.32)	0.649
GG	6 (2.4)	15 (5.3)	2.24	(0.85 - 5.86)	0.093
L769L allele					
T	397 (81.0)	446 (79.1)	0.89	(0.65 - 1.20)	0.432
G	93 (19.0)	118 (20.9)	1.13	(0.83 - 1.53)	
S836S genotype					
CC	228 (93.1)	245 (86.9)	0.49	(0.27 - 0.90)	0.020
CT	17 (6.9)	36 (12.8)	1.96	(1.07 - 3.59)	0.027
TT	0 (0.0)	1 (0.4)	-	-	-
S836S allele					
C	473 (96.5)	526 (93.3)	0.50	(0.28 - 0.89)	0.017
T	17 (3.5)	38 (6.7)	2.01	(1.12 - 3.61)	
S904S genotype					
CC	164 (66.9)	183 (64.9)	0.91	(0.64 - 1.31)	0.621
CG	74 (30.2)	86 (30.5)	1.13	(0.78 - 1.64)	0.509
GG	7 (2.9)	13 (4.6)	1.64	(0.64 - 4.19)	0.294
S904S allele					
C	402 (82.0)	452 (80.1)	0.88	(0.65 - 1.20)	0.433
G	88 (18.0)	112 (19.9)	1.13	(0.83 - 1.54)	
Haplotypes G691S/L769L/S836S/S904S	N=326	N =364			
G/T/C/C	235 (72.1)	253 (69.5)	0.88	(0.64 - 1.23)	0.457
G/G/C/C	66 (20.2)	62 (17.0)	0.81	(0.55 - 1.19)	0.279
A/T/C/G	14 (4.3)	24 (6.6)	1.57	(0.80 - 3.10)	0.190
G/T/T/C	4 (1.2)	2 (0.5)	0.45	(0.08 - 2.44)	0.351
A/T/C/C	4 (1.2)	5 (1.4)	1.12	(0.30 - 4.21)	0.866
G/T/C/G	2 (0.6)	7 (1.9)	3.18	(0.66 - 14.50)	0.151
G/G/T/C	1 (0.3)	10 (2.7)	9.18	(1.17 - 72.12)	0.035
A/G/C/C	0	1 (0.3)	-	-	-

n – Number; OR – Odds Ratio; CI – Confidence Interval

Looking at the single locus analysis, each of the minor alleles is overrepresented in the patients group; however these results are not all statistically significant.

Only the S836S SNP presented a statistically significant association with DTC; the CT genotype of this polymorphism is overrepresented in patients group compared to the control group, which is reflected in the overrepresentation of the minor allele of this polymorphism in allele analysis.

Regarding the haplotype analysis, 16 haplotypes were constructed using all possible genotypes combinations of the four studied *RET* polymorphisms, however only 8 haplotypes were observed in the studied populations. The GGTC haplotype is the only one with a statistically significant difference (p = 0.035), showing a 9.18-fold increased risk for DTC.

3.2.3.1. Analysis of cancer subtypes

The first subdivision made in the studied population was by cancer subtypes, so two patients subgroups were created, one with the PTC patients (n=249) and other with the FTC patients (n=33). Genotypic, allelic and haplotypic distributions of the studied polymorphisms in PTC and FTC were compared; data is represented in table 7.

Table 7 - Single locus and haplotypes analysis of *RET* polymorphisms in PTC and FTC patients.

Polymorphisms	PTC, n (%) N = 249	FTC, n (%) N = 33	OR	95% CI	p-value
G691S genotype					
GG	160 (64.3)	22(66.7)	0.90	(0.42 - 1.94)	0.786
GA	77 (30.9)	10(30.3)	1.03	(0.47 - 2.27)	0.942
AA	12 (4.8)	1(3.0)	1.62	(0.20 - 12.88)	0.645
G691S allele					
G	397 (79.7)	54 (81.8)	0.87	(0.45 - 1.69)	0.689
A	101 (20.3)	12 (18.2)	1.14	(0.59 - 2.22)	0.689
L769L genotype					
TT	159 (63.9)	20 (60.6)	1.15	(0.55 - 2.42)	0.716
TG	75 (30.1)	13 (39.4)	0.66	(0.31 - 1.40)	0.280
GG	15 (6.0)	0 (0.0)			
L769L allele					
T	393 (78.9)	53 (80.3)	0.92	(0.48 - 1.75)	0.795
G	105 (21.1)	13 (19.7)	1.09	(0.57 - 2.07)	0.795
S836S genotype					
CC	216 (86.7)	29 (87.9)	0.90	(0.30 - 2.73)	0.856
CT	32 (12.9)	4 (12.1)	1.07	(0.35 - 3.24)	0.906
TT	1 (0.4)	0 (0.0)	-	-	-
S836S allele					
C	464 (93.2)	62 (93.9)	0.88	(0.30 - 2.57)	0.815
T	34 (6.8)	4 (6.1)	1.14	(0.39 - 3.31)	0.815
S904S genotype					
CC	161 (64.7)	22 (66.7)	0.91	(0.42 - 1.97)	0.820
CG	76 (30.5)	10 (30.3)	1.01	(0.46 - 2.23)	0.980
GG	12 (4.8)	1 (3.0)	1.62	(0.20 - 12.88)	0.645
S904S allele					
C	398 (79.9)	54 (81.8)	0.88	(0.46 - 1.72)	0.716
G	100 (20.1)	12 (18.2)	1.13	(0.58 - 2.19)	0.716
Haplotypes G691S/L769L/S836S/S904S	N = 322	N = 42			
G/T/C/C	218 (67.7)	35 (83.3)	0.42	(0.18 - 0.98)	0.038
G/G/C/C	55 (17.1)	7 (16.7)	1.03	(0.44 - 2.44)	0.946
A/T/C/G	24 (7.5)	0	-	-	-
G/T/T/C	2 (0.6)	0	-	-	-
A/T/C/C	5 (1.6)	0	-	-	-
G/T/C/G	7 (2.2)	0	-	-	-
G/G/T/C	10 (3.1)	0	-	-	-
A/G/C/C	1 (0.3)	0	-	-	-

PTC – papillary thyroid carcinoma; FTC – follicular thyroid carcinoma; n – Number; OR – Odds Ratio; CI – Confidence Interval

In this single locus analysis no statistically significant difference between PTC and FTC patients was observed for genotype and allele distributions of *RET* polymorphisms. In haplotype analysis we found a significant under-representation of the GTCC haplotype in FTC patients compared to PTC patients. It is important to note that in FTC patients only two haplotypes were observed.

With the Bonferroni correction for multiple tests the significant p-value changed from 0.05 to 0.01, so the above results are not statistically significant.

3.2.3.2. Analysis of gender

A comparison between male and female subgroups was made for genotype, allele and haplotype frequencies of studied polymorphisms. Data is presented in table 8. Regarding the patients gender, no statistically significant differences were observed for single locus and haplotype analysis of RET polymorphisms.

Table 8 - Single locus analysis and haplotype analysis of *RET* polymorphisms in male and female patients.

Polymorphisms	Male, n (%) N = 47	Female, n (%) N = 235	OR	95% CI	p-value
G691S genotype					
GG	34 (72.3)	148 (63.0)	1.54	(0.77 - 3.07)	0.221
GA	9 (19.1)	78 (33.2)	0.48	(0.22 - 1.04)	0.057
AA	4 (8.5)	9 (3.8)	2.34	(0.69 - 7.93)	0.162
G691S allele					
G	77 (81.9)	374 (79.6)	1.16	(0.66 - 2.06)	0.605
A	17 (18.1)	96 (20.4)	0.86	(0.49 - 1.52)	0.605
L769L genotype					
TT	30 (63.8)	149 (63.4)	1.02	(0.53 - 1.95)	0.956
TG	16 (34.0)	72 (30.6)	1.17	(0.60 - 2.27)	0.646
GG	1 (2.1)	14 (6.0)	0.34	(0.04 - 2.68)	0.286
L769L allele					
T	76 (80.9)	370 (78.7)	1.14	(0.65 - 2.00)	0.643
G	18 (19.1)	100 (21.3)	0.88	(0.50 - 1.53)	0.643
S836S genotype					
CC	43 (91.5)	202 (86.0)	1.76	(0.59 - 5.22)	0.305
CT	4 (8.5)	32 (13.6)	0.59	(0.20 - 1.76)	0.338
TT	0 (0.0)	1 (0.4)	-	-	-
S836S allele					
C	90 (95.7)	436 (92.8)	1.75	(0.61 - 5.07)	0.293
T	4 (4.3)	34 (7.2)	0.57	(0.20 - 1.65)	0.293
S904S genotype					
CC	33 (70.2)	150 (63.9)	1.34	(0.68 - 2.64)	0.403
CG	10 (21.3)	76 (32.3)	0.57	(0.27 - 1.20)	0.133
GG	4 (8.5)	9 (3.8)	2.34	(0.69 - 7.93)	0.162
S904S allele					
C	76 (80.9)	376 (80.0)	1.06	(0.60 - 1.85)	0.850
G	18 (19.1)	94 (20.0)	0.95	(0.54 - 1.66)	0.850
Haplotypes G691S/L769L/S836S/S904S	N = 72	N = 292			
G/T/C/C	49 (68.1)	204 (69.9)	0.92	(0.53 - 1.60)	0.765
G/G/C/C	12 (16.7)	50 (17.1)	0.97	(0.49 - 1.93)	0.926
A/T/C/G	8 (11.1)	16 (5.5)	2.16	(0.88 - 5.26)	0.085
G/T/T/C	1 (1.4)	1 (0.3)	4.10	(0.25 - 66.33)	0.282
A/T/C/C	0	5 (1.7)	-	-	-
G/T/C/G	2 (2.8)	5 (1.7)	1.64	(0.31 - 8.63)	0.555
G/G/T/C	0	10 (3.4)	-	-	-
A/G/C/C	0	1 (0.3)	-	-	-

n – Number; OR – Odds Ratio; CI – Confidence Interval

3.2.3.3. Analysis of age at diagnosis

The patients' population was subdivided according to age at diagnosis; two subgroups were created with a cutoff at 45 years. The comparison between these groups for single locus and haplotype frequencies is presented in table 9.

Table 9 - Effect of *RET* polymorphisms and haplotypes on age at diagnosis of DTC.

Polymorphisms	≤ 45 years, n (%) N = 122	> 45 years, n (%) N = 160	OR	95% CI	p-value
G691S genotype					
GG	71 (58.2)	111 (69.4)	0.61	(0.38 - 1.01)	0.052
GA	48 (39.3)	39 (24.4)	2.01	(1.21 - 3.36)	0.007
AA	3 (2.5)	10 (6.3)	0.38	(0.10 - 1.40)	0.133
G691S allele					
G	190 (77.9)	261 (81.6)	0.80	(0.53 - 1.20)	0.278
A	54 (22.1)	59 (18.4)	1.26	(0.83 - 1.90)	0.278
L769L genotype					
TT	80 (65.6)	99 (61.9)	1.17	(0.72 - 1.92)	0.523
TG	34 (27.9)	54 (33.8)	0.76	(0.45 - 1.27)	0.291
GG	8 (6.6)	7 (4.4)	1.53	(0.54 - 4.35)	0.418
L769L allele					
T	194 (79.5)	252 (78.8)	1.05	(0.69 - 1.58)	0.826
G	50 (20.5)	68 (21.3)	0.96	(0.63 - 1.44)	0.826
S836S genotype					
CC	106 (86.9)	139 (86.9)	1.00	(0.50 - 2.01)	0.998
CT	15 (12.3)	21 (13.1)	0.93	(0.46 - 1.89)	0.836
TT	1 (0.8)	0 (0.0)	-	-	-
S836S allele					
C	227 (93.0)	299 (93.4)	0.94	(0.48 - 1.82)	0.849
T	17 (7.0)	21 (6.6)	1.07	(0.55 - 2.07)	0.849
S904S genotype					
CC	72 (59.0)	111 (69.4)	0.64	(0.39 - 1.04)	0.071
CG	45 (36.9)	41 (25.6)	1.70	(1.02 - 2.83)	0.042
GG	5 (4.1)	8 (5.0)	0.81	(0.26 - 2.55)	0.721
S904S allele					
C	189 (77.5)	263 (82.2)	0.74	(0.49 - 1.13)	0.163
G	55 (22.5)	57 (17.8)	1.34	(0.89 - 2.03)	0.163
Haplotypes G691S/L769L/S836S/S904S	N = 142	N = 222			
G/T/C/C	98 (69.0)	155 (69.8)	0.96	(0.61 - 1.52)	0.871
G/G/C/C	22 (15.5)	40 (18.0)	0.83	(0.47 - 1.47)	0.532
A/T/C/G	7 (4.9)	17 (7.7)	0.63	(0.25 - 1.55)	0.306
G/T/T/C	0	2 (0.9)	-	-	-
A/T/C/C	2 (1.4)	3 (1.4)	1.04	(0.17 - 6.32)	0.964
G/T/C/G	6 (4.2)	1 (0.5)	0.36	(0.04 - 3.02)	0.326
G/G/T/C	7 (4.9)	3 (1.4)	3.79	(0.96 - 14.89)	0.042
A/G/C/C	0	1 (0.5)	-	-	-

n – Number; OR – Odds Ratio; CI – Confidence Interval

When the statistical significance was adjusted, for the number of comparisons, to a p-value <0.01, only the overrepresentation of G691S polymorphism GA genotype remained statistically significant.

3.2.3.4. Analysis of tumor size

Another parameter taken into account in this study was the size of the tumor at diagnosis; therefore the patients group was subdivided in two subgroups with a cutoff at 10 mm of tumor diameter. Table 10 summarizes the genotype, allele and haplotype distributions in the subgroups and presents the OR for each studied polymorphism with a confidence interval of 95%.

The analysis of the distribution of the genotypes of the *RET* polymorphisms according to the tumor size at diagnosis, showed only a significant overrepresentation of the G691S

heterozygous genotype in the patients group with tumors greater than 10 mm of diameter at diagnosis.

No significant differences were found between the two groups of patients for the haplotypes distribution, since all p-values were greater than 0.05. When the adjustment for multiple tests was performed by Bonferroni correction, statistical significance was adjusted to a p-value <0.01, and thus the overrepresentation of the G691S heterozygous genotype were not statistically significant.

Table 10 - Effect of *RET* polymorphisms and respective haplotypes on tumor size at age of diagnosis.

Polymorphisms	Size≤10 mm, n (%) N = 81	Size > 10 mm, n (%) N = 201	OR	95% CI	p-value
G691S genotype					
GG	58 (71.6)	124 (61.7)	1.57	(0.89 - 2.74)	0.115
GA	17 (21.0)	70 (34.8)	0.50	(0.27 - 0.91)	0.023
AA	6 (7.4)	7 (3.5)	2.22	(0.72 - 6.81)	0.155
G691S allele					
G	133 (82.1)	318 (79.1)	1.21	(0.76 - 1.93)	0.421
A	29 (17.9)	84 (20.9)	0.83	(0.52 - 1.32)	0.421
L769L genotype					
TT	47 (58.0)	132 (65.7)	0.72	(0.43 - 1.23)	0.228
TG	30 (37.0)	58 (28.9)	1.45	(0.84 - 2.50)	0.180
GG	4 (4.9)	8 (5.5)	0.90	(0.28 - 2.90)	0.856
L769L allele					
T	124 (76.5)	322 (80.1)	0.81	(0.52 - 1.26)	0.347
G	38 (23.5)	80 (19.9)	1.23	(0.80 - 1.91)	0.347
S836S genotype					
CC	70 (86.4)	175 (87.1)	0.95	(0.44 - 2.02)	0.885
CT	11 (13.6)	25 (12.4)	1.11	(0.52 - 2.37)	0.795
TT	0 (0.0)	1 (0.5)			
S836S allele					
C	151 (93.2)	375 (93.3)	0.99	(0.48 - 2.04)	0.975
T	11 (6.8)	27 (6.7)	1.01	(0.49 - 2.09)	0.975
S904S genotype					
CC	58 (71.6)	125 (62.2)	1.53	(0.88 - 2.69)	0.134
CG	19 (23.5)	67 (33.3)	0.61	(0.34 - 1.11)	0.103
GG	4 (4.9)	9 (4.5)	1.11	(0.33 - 3.71)	0.867
S904S allele					
C	135 (83.5)	317 (78.9)	1.34	(0.83 - 2.16)	0.228
G	27 (16.7)	85 (21.1)	0.75	(0.46 - 1.20)	0.228
Haplotypes G691S/L769L/S836S/S904S	N = 116	N = 248			
G/T/C/C	76 (65.5)	177 (71.4)	0.76	(0.48 - 1.22)	0.258
G/G/C/C	23 (19.8)	39 (15.7)	1.33	(0.75 - 2.34)	0.332
A/T/C/G	9 (7.8)	15 (6.0)	1.31	(0.55 - 3.08)	0.540
G/T/T/C	1 (0.9)	1 (0.4)	2.15	(0.13 - 34.64)	0.581
A/T/C/C	3 (2.6)	2 (0.8)	3.27	(0.54 - 19.82)	0.174
G/T/C/G	1 (0.9)	6 (2.4)	0.36	(0.04 - 3.02)	0.326
G/G/T/C	2 (1.7)	8 (3.2)	0.53	(0.11 - 2.52)	0.414
A/G/C/C	1 (0.9)	-	-	-	-

n – Number; OR – Odds Ratio; CI – Confidence Interval

4. Discussion and conclusion

The hypothesis tested in this case-control study was that four common polymorphisms of the *RET* proto-oncogene are associated with the risk of developing thyroid cancer.

The present study focused on thyroid cancer patients with DTC (PTC or FTC). These carcinomas subtypes were chosen due to the fact that the role of these *RET* polymorphisms in MTC have already been studied in several populations including the Portuguese population [26, 69]. Another aspect is associated with the difficulty to obtain a reasonable MTC patients population due to its low incidence, at least there are no studies of these dimensions, and moreover DTC represents the majority of thyroid cancer.

The control group consisted of 245 Portuguese Caucasian individuals which was in HWE for all studied polymorphisms. this allows to assume that the sample is large enough, mating is random, allelic frequencies are conserved from generation to generation, no significant migrations occur and mutation, selection, genetic drift and gene flow are negligible [80]. In our series, polymorphisms frequencies observed in normal individuals were within the range of variation defined in the European population, except for the L769L polymorphism.

The study of polymorphic variants raises critical questions, and one important aspect is whether or not these variants are in LD. LD refers to the phenomenon that alleles that are close together in the genome tend to be inherited together. Since this study depicts polymorphisms of the same gene and with little distance (5 kb at maximum) between them, high pairwise linkage disequilibrium coefficients were expected. However, this did not occur and the strongest LD was verified between the G691S and S904S polymorphisms (D' = 0.94; r^2 = 0.89), that are physically the most distant polymorphisms in the study. It was also observed that the L769L and S836S polymorphisms are in moderate LD (D' = 0.64; r^2 = 0.06). This phenomenon is not well clarified, but it may be associated with processes of gene conversion. high gene conversion rates in humans has already been shown and it has been proved that this is important in LD between tightly linked genetic markers [81]. At some time in the evolution of the population, a short stretch of chromosome including the L769L and S836S polymorphisms could have been inserted between G691S and S904S polymorphism, this would justify the strong LD between G691S and S904S polymorphism and the absence of LD with the other two polymorphisms. Our results are similar with other studies [82, 83] for the LD between G691S and S904S polymorphisms, although in other studies this LD is complete (D'=1). Regarding LD between the L769L and S836S polymorphisms, the findings in this study are in agreement with the described by Lesueur et al. (2002) and Ceolin et al. (2012), however they found other associations that were not found in the present study [76, 83]. Differences in the analysis of LD among the studies may be explained in part by the genetic background of different populations.

In the present study, we analyzed four *RET* polymorphism and the only polymorphism that results in aminoacidic variation is the G691S located in exon 11. It is possible to assume that

this aminoacid modification has a cooperative effect in RET dimerization or form a new phosphorylation site on the intracellular domain of the RET receptor and it could be associated with susceptibility to a disease [68] as seen in many studies conducted on MTC [66, 67].

The other studied polymorphisms are silent and therefore do not leads to an aminoacidic change, so the mechanism by which these polymorphisms may act to increase the risk for diseases remains to be identified, even though many proposals have already been described. Three principal hypothesis have been speculated: i) silent polymorphisms may influence RNA splicing conducting to instability in protein synthesis; ii) silent polymorphisms could be in LD with another unknown functional nucleotide variant [75]; iii) silent polymorphisms could make the gene more vulnerable to damages by environmental factors, like radiation [14]. Several studies have been developed in order to prove by functional tests these hypothesis for silent *RET* polymorphisms, but until now the results have been negative, so these hypothesis are still only speculative [84, 85].

In this study, we started by comparing all the patients with the control group. This analysis was performed, to assess if any one of the genotypes, alleles or haplotypes of *RET* polymorphisms are distributed in a significantly different manner in patients compared to the general population and thus find a predisposition for DTC in a low-penetrance manner.

The lack of association with DTC was verified for the non-synonymous G691S polymorphism. The frequencies of the L769L polymorphism were similar in both patients and controls, thus suggesting the absence of a predisposing role to the development of DTC. This is in agreement with data by Ho et al. (2005) [14]. These findings contrast with a study developed by Lesueur et al. (2002) which associated the L769L polymorphism with the PTC phenotype. In that study 4 populations from different countries were evaluated (one was Portuguese) Lesueur et al (2002) also found an association between PTC and the silent A45A polymorphism located in exon 2 [76]. This polymorphism was not assessed in our study, however Borrego et al. (1999) [84] proposed that this variant leads to alternative splicing which results in a protein with a deletion in the extracellular domain. These two polymorphisms (L769L and A45A) exhibit a strong association with Hirchsprung disease [84, 86].

The T allele of the S836S polymorphism is significantly overrepresented in DTC patients compared to the control population (OR 2.01 95% CI 1.12-3.61), so this allele is associated with the increased risk of developing DTC in the studied population. This finding is in concordance with Ho et al. that noticed a possible association of S836S polymorphism with an increased risk of DTC [14]. Curiously, this same allele was underrepresented, in the study developed by Lesueur et al. (2002), in familial PTC [76].

Since a polymorphism could be associated with a particular parameter or characteristic, four different patient parameters were evaluated: cancer subtype, gender, age at diagnosis, and tumor size at diagnosis.

Although PTC and FTC both derive from follicular cells they have many differences particularly in molecular genetics, so it is important to take into account this division when studying the role of polymorphisms in this cancer. It is well established that PTC and FTC are more prevalent in females than in males, so it is important to see if *RET* polymorphisms could affect gender in a different way. However, among patients, genotypic, allelic and haplotypic distribution according to the gender did not disclose any significant differences between females and males.

The age at diagnosis is an important parameter to take in account. The reason why the same type of cancer affects people with very different ages is a question that still leads to many studies. In this study we intended to know if some of the studied *RET* polymorphisms influence the age at which an individual develops thyroid cancer. Regarding the age of cancer onset, it is also expected that if there is a polymorphism increasing the risk for cancer, the cancer is more likely to develop at an earlier age than cancer that is solely due to environmental factors.

The patients and controls were divided in two groups with a 45 years old cutoff. This cut off was chosen because this is the age used as prognosis criteria in the most common cancer staging system (Tumor-Node-Metastases (TNM) classification) [87]. Allelic and genotypic distribution of the silent L769L and S836S polymorphisms, according to the age at diagnosis of DTC (≤ 45 years or > 45 years) did not disclose any significant differences. The other two polymorphisms showed statistically significant overrepresentations of their heterozygous genotypes. The fact of the G691S and S904S heterozygous genotypes are both overrepresented may is due to the LD between them. No other study of DTC has taken into account this parameter and at the moment there is no explanation for these findings.

In this study, another parameter evaluated was the size of the tumor at diagnosis; the chosen cutoff was 10 mm of diameter, since this is the size that differentiates microcarcinomas from larger thyroid carcinomas. The microcarcinomas are usually associated with a lack of clinical significance, however in some cases they represent a precursor of the larger tumor [20]. It is important to note that the increase in cases of PTC around the world in recent years is due largely to micro-PTC [24]. Taking all this into account, this division between carcinomas sizes at diagnosis is relevant. In our study, the heterozygous genotype of G691S polymorphism revealed an overrepresentation in patients harboring tumors larger then 10mm of diameter at diagnosis. Until now, no other study of DTC has taken into account tumor size parameter so, there is no explanation for this finding.

A cluster of DNA variations or polymorphisms that are transmitted together over generations is designated as haplotype. Haplotypes represent a more discriminative state of a chromosomal region; therefore a haplotype analysis may be more powerful than a single locus analysis. It is understandable that for some regions, the haplotypes, rather than individual polymorphisms, are functional units and thus for these regions, stronger associations may be detected by performing haplotype analysis. Furthermore a haplotype may amplify the effects of a single polymorphism, in other words, haplotypes can represent a synergistic effect of the variants that compose it [64].

With the four studied polymorphisms, a total of 8 haplotypes of *RET* were observed in the patients and control groups. Some of the haplotypes displayed different frequencies between cases and controls indicating putative associations with the disease, although the majority was not significant.

When the haplotypes of the DTC group were analyzed a significant overrepresentation of the GGTC haplotype was found compared to the control group. This haplotype showed an overrepresentation also in patients diagnosed over 45 years old. This haplotype includes the T allele of the S836S polymorphism that is associated with an increased risk in the single locus analysis. It also includes the rare allele of the L769L polymorphism and this may represent a synergistic effect caused by the presence of these two rare alleles together. The GTCC haplotype proved to be significantly underrepresented in PTC when compared to FTC, this finding may be due to the small size of the FTC group.

For several reasons, the results of this study should be viewed with caution. First, given the relatively small sample size, it is possible that the results are caused by chance and would need to be confirmed with a larger sample population. In addition there are possible confounding genetic and environmental factors that have not been adjusted. Moreover, the study may have suffered from selection bias that could have resulted in a study population that does not necessarily reflect the genetic characteristics of PTC and FTC found in the general population. Since multiple comparisons were performed in this study, the Bonferroni correction was applied and showed that the results discussed above are not necessarily statistically significant. However for tightly linked polymorphisms, as in this study, the Bonferroni correction is conservative [81]. Therefore it could underestimate potentially relevant results, taking into account that variants are expected to have a low or moderate effect for this cancer.

In conclusion, our data suggest that the S836S polymorphism in exon 14 may be associated with an increased risk of DTC. Also the heterozygous genotype of G691S/S904S polymorphisms seems to be associated with age of onset of DTC and additionally the heterozygous genotype of G691S polymorphism appeared to be in association with tumor size. The GGTC haplotype appears to be associated with increased risk of DTC particularly in those developed in later

age (after the age of 45 years). Nevertheless, these findings need to be confirmed by larger studies in order to re-evaluate the role of these variants in the susceptibility to DTC.

5. References

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