THE GENETICS OF PAPILLARY MICROCARCINOMAS OF THE THYROID

Gustavo Branquinho Crespo Teixeira Santos

DISSERTAÇÃO DE MESTRADO APRESENTADA AO INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR DA UNIVERSIDADE DO PORTO EM ONCOLOGIA

GUSTAVO BRANQUINHO CRESPO TEIXEIRA SANTOS

THE GENETICS OF PAPILLARY MICROCARCINOMAS OF THE THYROID

Dissertação de candidatura ao grau de Mestre em Oncologia – Especialização em Oncologia Molecular submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientadora - Professora Doutora Ana Paula Soares Dias Ferreira
Categoria – Professora Auxiliar (FMUP), Coordenadora do Grupo Cancer Signaling and Metabolism (I3S/IPATIMUP)
Afiliações – Faculdade de Medicina da Universidade do Porto, Instituto de Investigação e Inovação em Saúde/Instituto de Patologia e Imunologia Molecular da Universidade do Porto

Co-orientador – Doutor João Pedro Rico de Oliveira Vinagre
Categoria – Investigador pós-doutoramento no grupo Cancer Signaling and Metabolism (I3S/IPATIMUP)
Afiliações – Instituto de Ciências Biomédicas Abel Salazar, Instituto de Investigação e Inovação em Saúde/Instituto de Patologia e Imunologia Molecular da Universidade do Porto

AGRADECIMENTOS

Em primeiro lugar gostaria de agradecer ao Instituto de Patologia e Imunologia Molecular da Universidade do Porto/ Instituto de Investigação e Inovação em Saúde por me ter dado a oportunidade de realizar a minha tese de mestrado num local com reconhecimento internacional na área da investigação oncológica.

À Professora Doutora Paula Soares por me ter dado a oportunidade de realizar este trabalho no seu grupo de investigação, por ter orientado esta tese e me ter conduzido neste percurso.

Ao Doutor João Vinagre por me ter recebido tão bem e me ter deixado sempre à vontade. Obrigado por todos os ensinamentos e conhecimentos transmitidos.

Ao grupo Cancer Signaling and Metabolism por me terem recebido bem e me terem ajudado sempre que precisei. Um obrigado especial ao João Pedro Amorim, amigo e colega de curso e bancada, que me ajudou nas tarefas do dia-a-dia e me acompanhou em todo o percurso.

À minha família. À minha tia Madalena por me ter ajudado na adaptação a uma nova cidade. Aos meus pais, a quem devo tudo aquilo que sou, por me terem sempre motivado e percebido o valor do investimento na formação académica. Aos meus irmãos, Miguel e Guilherme, por estarem sempre presentes e me acompanharem em todas as etapas da minha vida.

À minha namorada, Mariana Valente, porque mesmo longe me fez sentir sempre perto de casa. Obrigado por me teres ajudado nestes 2 anos em que vivi longe dos meus amigos e família.

A todos aqueles que não referi mas que foram igualmente importantes, muito obrigado!

INDEX

RESUMO

Os microcarcinomas papilares da tiróide (mPTC) são definidos como carcinomas papilares da tiróide (PTC) que medem 1 centímetro ou menos na sua máxima dimensão. Estes tumores são muito comuns e são, muitas vezes, encontrados incidentalmente. As taxas de incidência dos mPTC têm aumentado constantemente em todo o mundo devido à melhoria dos métodos de diagnóstico. Na maioria dos casos, os mPTC têm um comportamento benigno com percurso indolente e um prognóstico excelente. No entanto, alguns casos apresentam características de agressividade e podem requerer tratamentos mais agressivos. A estratégia terapêutica padrão para estes tumores continua a ser um tema controverso. Com o objectivo de prever os casos que vão causar doença significativa muitos estudos têm tentado associar marcadores de mau prognóstico com alterações genéticas neste tipo de tumor.

Os PTC frequentemente têm alterações genéticas que levam à activação da via de sinalização mitogen-activated protein kinase (MAPK). Essas alterações incluem as mutações pontuais do proto-oncogene B-Raf (BRAF) e RAS Viral Oncogene Homolog (RAS) e o rearranjo *RET/PTC*. As mutações envolvendo um destes genes são encontradas em 70% dos PTC e raramente se sobrepõe no mesmo tumor. Recentemente foram encontradas mutações no promotor do gene *telomerase reverse transcriptase* (*TERT*), com uma prevalência de 10% em cancro da tiróide.

O objectivo principal deste trabalho era avaliar o perfil genético dos mPTC. Para isso, planeámos avaliar a prevalência das mutações do *BRAF* (exão 15, região do codão 600), *NRAS* (exão 3, região do codão 61) e promotor do *TERT*, numa série de mPTC. Também planeámos investigar as possíveis associações entre estas alterações genéticas com características clinicopatológicas clássicas.

A caracterização genética desta série revelou a ausência de mutações no gene NRAS. Foi também possível ver, como esperado, um grande número de casos multifocais, incidentais e um maior número de casos a afectar as mulheres do que os homens. Curiosamente, a arquitectura folicular foi predominante nesta série e isso poderá estar relacionado com as características de agressividade destes casos. O trabalho apresentado nesta tese é parte de um trabalho que ainda está a decorrer, com mais casos para serem analisados em relação às alterações genéticas do BRAF, NRAS e promotor do TERT. Os resultados que vierem desta análise futura são de grande importância para melhor perceber a relação entre as alterações genéticas e o resultado clínico neste tipo de tumores. Continua por ser encontrado um marcador que consiga distinguir quais dos mPTC vão de facto apresentar um mau prognóstico e requerer uma

abordagem mais radical para os tratar. Para além disso, como observado em alguns países, o aumento dos casos de detecção destas pequenas lesões representa um grande encargo económico. Assim, é fundamental identificar marcadores que nos permitam reconhecer os casos que vão causar doença significativa para que uma atitude terapêutica mais adequada seja tomada.

ABSTRACT

Papillary microcarcinoma of the thyroid (mPTC) is defined as a papillary thyroid carcinoma (PTC) that measures 1 centimeter or less in its maximum dimension. These tumors are very common and are often found incidentally. mPTC incidence rates have been steadily increasing all over the world due to improvement of diagnostic methods. In most of the cases, mPTC has a benign behaviour with an indolent course and excellent prognosis. However, some have aggressive features and may require aggressive treatment. The standard therapeutic approach for these tumors remains controversial. With the purpose of predicting those that will cause significant disease, many studies have been trying to associate poor prognostic markers with genetic alterations in this type of cancer.

PTC frequently has genetic alterations leading to the activation of the mitogen-activated protein kinase (MAPK) signalling pathway. Those include B-Raf Proto-Oncogene, Serine/Threonine Kinase (BRAF) and RAS Viral Oncogene Homolog (RAS) point mutations and *RET/PTC* rearrangements. Mutations involving one of these genes are found in >70% of PTC and they rarely overlap in the same tumor. Recently, mutations in *telomerase reverse transcriptas*e (*TERT*) gene promoter were found in thyroid cancer with an overall prevalence of near 10%.

The general aim of this work was to evaluate the mPTC genetic profile. In order to do that, we intended to evaluate the prevalence of *BRAF* (exon 15, codon 600 region), *NRAS* (exon 3, codon 61 region) and *TERT* promoter mutations in a series of mPTC. We also intended to investigate possible associations of those genetic alterations with classical clinicopathological features.

The genetic characterization of this series revealed no mutations in NRAS gene. It was also possible to see a high number of multifocal, incidental and female cases, as expected. Interestingly, follicular architecture was predominant in this series and that can be related with the aggressive features of these cases. The work presented on this thesis is still part of an ongoing work with more cases to be analysed for genetic alterations in *BRAF, NRAS* and *TERT* promoter. The results arising from this future analysis are of major importance for better understanding the relationship between genetic alterations and clinical outcome in this type of tumors. It remains to be found a marker that could distinguish which of these mPTC will indeed present a worse prognosis and will require a more radical approach in order to treat them. Additional, as observed in some countries, the increased detection of these small lesions represents an economic burden. Therefore,

we aim to identify those that will cause significant disease so an aggressive therapeutic approach can be taken.

List of figures

List of tables

Abbreviations

-124 G>A	Guanine to Adenine conversion at position -124
-148 G>A	Guanine to Adenine conversion at position -148
10q11.2	Chromosome 10, long arm, region 1, band 1, sub-band 2
7q34	Chromosome 7, long arm, region 3, band 4
99mTc	Technetium-99m
AKAP9	A-kinase anchor protein 9
AKT	Protein Kinase B
ATC	Anaplastic thyroid cancer
ATP	Adenosine triphosphatase
bp	Base pairs
BRAF	B-Raf Proto-Oncogene/ v-Raf murine sarcoma viral oncogene homolog B
c.182 A>G	Adenine to guanine conversion at position 182
c-Kit	Proto-oncogene c-Kit/ stem cell growth factor receptor/ CD117
c-mPTC	Classical variant of papillary thyroid microcarcinoma
C>T	Cytosine do thymine conversion
CHSJ	Centro Hospitalar de São João
ºC	Celsius degree
cm	centimeter
DIT	Di-iodotyrosine
DNA	Deoxyribonucleic acid
DTC	Differentiated thyroid cancer
EBRT	External Beam Radiation Therapy
ERKs	Extracellular signal-regulated kinases
ETS	E-twenty six transformation-specific transcription factors family
FFPE	Formalin-fixed paraffin-embedded
FNAB	Fine-needle aspiration biopsy
FTC	Follicular thyroid carcinoma

fv-mPTC	Follicular variant of papillary thyroid microcarcinoma
g	Gram
G-rich	Guanine rich
GDP	Guanine diphosphate
GGAA	Sequence of two guanines and two adenines
GTP	Guanine triphosphate
GTPase	GTP hydrolase enzyme
G465	Glycine at position 465
G597	Glycine at position 597
H&E	Haematoxylin and Eosin
HRAS	Harvey Rat Sarcoma Viral Oncogene Homolog
I^-	Iodide
I^0	Iodine
I^{123}	Iodine-123 radioisotope
I^{131}	Iodine-131 radioisotope
K-Da	Kilodalton
Kb	Kilobases (base pairs)
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
mm	milimeter
MNG	Multinodular goiter
mPTC	Papillary thyroid microcarcinoma
MTC	Medullary thyroid carcinoma
Na^+	Sodium
NCOA4 (ELE1)	Nuclear receptor coactivator 4
NIS	Sodium-Iodide Symporter
NRAS	Neuroblastoma RAS Viral Oncogene Homolog
PCR	Polymerase chain reaction
PDGFRA	Platelet-derived growth factor receptor alpha

PDTC	Poorly differentiated thyroid carcinoma
PI3K	Phosphoinositide 3-kinase
PTC	Papillary Thyroid Cancer
Q61R	Glutamine to Arginine conversion at position 61
RAI	Radioactive iodine therapy
RAS	Rat Sarcoma Viral Oncogene
RET	Proto-oncogene *RET* (*rearranged during transfection)*
RORENO	Registo Oncológico Regional do Norte
T1799A	Thymine to Adenine conversion at position 1799
T_3	Tri-iodothyronine
T_4	Thyroxine
TC	Thyroid cancer
TERT	Telomerase reverse transcriptase gene
TK	Tyrosine Kinase
TKI	Tyrosine Kinase Inhibitor
TPO	Thyroid Peroxidase
TSH	Thyroid Stimulating Hormone
µl	Microliter
V472	Valine at position 472
V600E	Valine to Glutamate conversion at position 600
V601	Valine at position 601
VEGFR	Vascular Endothelial Growth Factor Receptor

INTRODUCTION

INTRODUCTION

THE THYROID GLAND

Thyroid anatomy

The thyroid gland (Figure 1) is composed of two lobes connected by an isthmus and is located in the trachea approximately at the level of the second tracheal ring [3]. Each lobe is about 4 cm in length and 2 cm thickness and resides in a bed between the trachea and larynx medially and the carotid sheath and the sternocleidomastoid muscles laterally [3, 4]. The strap muscles are anterior to the thyroid lobes, and the parathyroid glands and recurrent laryngeal nerves are associated with the posterior surface of each lobe [3]. The gland is enveloped by the deep cervical fascia and is attached firmly to the trachea by the ligament of Berry [3]. Weighing approximately 20g, is one of the largest endocrine glands in the body and receives a high blood supply from the superior and inferior thyroid arteries [4]. A rich plexus of lymph vessels is in close proximity of the individual follicles, but no unique role in the thyroid function has been assigned to the system [5]. The major, if not only, secretory pathway for thyroid hormone is through the venous drainage of the thyroid rather than through the lymphatics, nonetheless thyroglobulin is mainly secreted in the lymph [5].

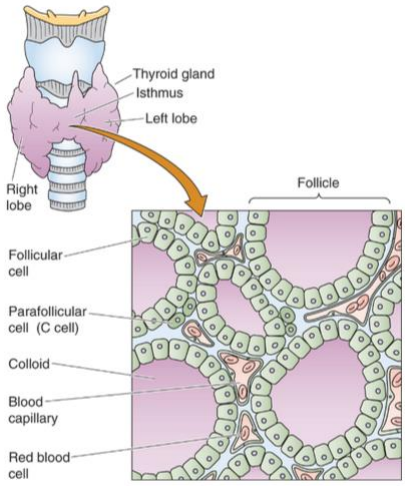

Figure 1 - Macro and microscopic structure of the thyroid gland. The gland is located in the lower anterior neck across the front of the trachea. A single layer of follicular cells outlines each of the basic units, the follicle, and colloid is present in their lumen. In the interfollicle space there can be seen blood vessels and a couple of C cells grouped together *(Boron et al. (2012) Section VIII: The endocrine system, in Medical Physiology: A cellular and molecular approach).*

The adult thyroid is composed of follicles, or acini, that are considered as the primary and secretory units of the organ. The cells of the follicles participate in hormone synthesis: tri-iodothyronine (T_3) and tetra-iodothyronine or thyroxine (T_4); and the lumina are the storage depots. In the normal adult gland, the follicles are roughly spherical and vary considerably in size with an average diameter of 300 microns. Their walls consist of a continuous epithelium one cell deep, the parenchyma of the thyroid. Within the follicle and filling its lumen is the homogeneous colloid, composed of a mixture of proteins, principally thyroglobulin [5].

In addition to the acinar cells, there are individual cells or small groups of cells which may appear as clusters between follicles. These light cells, or C-cells, are a distinct category that was believed to be derived from the neural crest via the ultimobranchial body; recently, the neural crest-derived theory has been challenged and an endoderm stem cell-derived differentiation has been proposed [6]. These cells secrete calcitonin in response to an increase in serum calcium [5].

Outside the follicles other types of cells populate the thyroid: the endothelial cells, the fibroblasts and immune cells [5].

Thyroid Physiology

The synthesis of thyroid hormones (Figure 2) is a complex process that occurs between the follicle cells and the colloid. Iodide is actively transported by the Sodium-Iodide (Na^+/I^-) symporter (NIS) against an electrical gradient at the basal membrane of follicular cells [5]. There, it is oxidized to active iodine in a reaction catalysed by thyroid peroxidase (TPO) [4]. At the apical-colloid interface, iodine is immediately incorporated into the tyrosine residues of thyroglobulin molecules [4]. Thyroglobulin is a large glycoprotein synthesized in follicular cells and about one quarter of its residues can be iodinated [4]. Once iodinated, thyroglobulin is taken up into the colloid of the follicle where a coupling reaction, catalysed by TPO, between pairs of iodinated tyrosine molecules occurs [4]. The coupling of two tyrosine residues each iodinated at two positions (di-iodotyrosine, DIT) produces T_4, whereas the combination of DIT with mono-iodotyrosine produces T_3 [4]. Thyroid hormones are therefore stored in this state and are only released when the thyroglobulin molecule is uptake into the follicular cells [4]. Secretion of thyroid hormones is stimulated by the thyroid stimulating hormone (TSH), also called thyrotropin, and requires endocytosis of thyroglobulin, its hydrolysis and release of thyroid hormones

from the cell [5]. The active uptake of iodide appears to be the main control point for hormone synthesis and is stimulated by TSH [4].

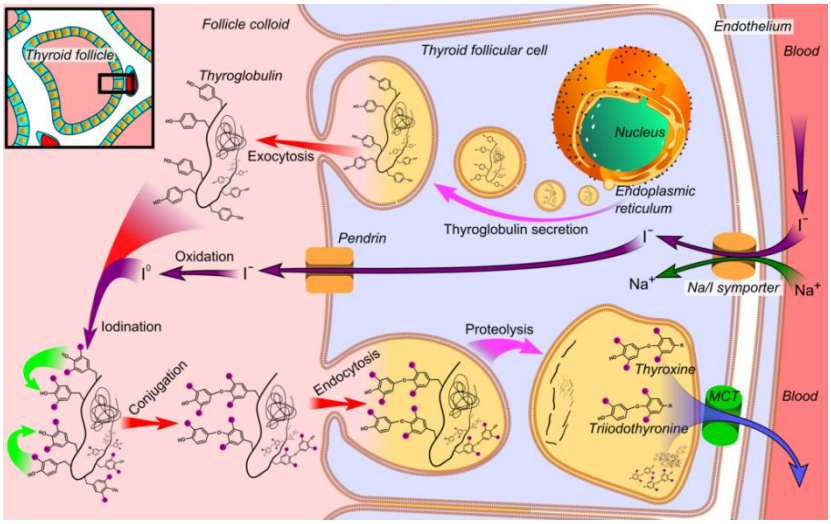

Figure 2 - Synthesis of thyroid hormones as seen on an individual thyroid follicular cell - Thyroglobulin is synthesized in the endoplasmic reticulum and secreted into the lumen of thyroid follicle by exocytosis. At the same time, iodide (I^-) is brought into the cell by a sodium-iodide (Na^+/I^-) symporter. This iodide is brought out of the follicular cells and into the lumen by the transporter pendrin. In the lumen, iodide (I^-) is oxidized to iodine (I^0) by an enzyme called thyroid peroxidase. Iodine (I^0) is very reactive and iodinates the thyroglobulin at tyrosyl residues. Adjacent tyrosyl residues are paired together. The entire complex re-enters the follicular cell by endocytosis. Proteolysis liberates thyroxine and triiodothyronine molecules, which enter the bloodstream [1] *(Häggström et al (2014.) Medical gallery of Mikael Häggström 2014, in Wikiversity Journal of Medicine).*

THYROID TUMORS

Thyroid carcinoma represents a very heterogeneous disease being composed of distinct and clinically different entities, namely differentiated thyroid carcinoma (DTC), poorly differentiated thyroid cancer (PDTC), anaplastic thyroid carcinoma (ATC), and medullary thyroid carcinoma (MTC) [7] [8]. Based in tumour morphology DTC can be further stratified in papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC) [9]. ATC, arising from follicular cells comprehend the rarest but the most lethal thyroid cancer affecting elderly patients and being characterized by an high aggressiveness and a very poor prognosis [10]. Poorly differentiated thyroid carcinomas and ATC are believed to mainly develop as a result of dedifferentiation of a well-differentiated papillary or follicular thyroid carcinoma [7]. MTC represents a neuroendocrine tumor derived from the parafollicular C-cells of the thyroid gland and is irresponsive to radioiodine therapy (RAI) [8] [11].

Presentation, diagnosis and treatment

At the time of initial assessment, most patients with thyroid cancer have a palpable neck mass, either a primary intrathyroidal tumor or metastatic regional lymphadenopathy [12]. In some patients, however, the tumor may be clinically occult, and the impalpable lesion may first be recognised on a high-resolution neck imagiological study or at the time of surgical intervention for presumed benign thyroid disease [12]. Therefore, thyroid ultrasonography is the first imaging study to perform in any patient with a possible thyroid malignancy [13]. After identification of a lesion by ultrasound imaging, fine-needle aspiration biopsy (FNAB) is performed [13]. FNAB is considered the best first-line diagnostic procedure for a thyroid nodule, being a minimally invasive procedure, safe and with near 80% of sensitivity and near 100% of specificity [14, 15]. FNAB will allow cytological or histological analysis for diagnostic confirmation [12]. Before the advent of FNAB, thyroid scintigraphy performed with Technetium Tc 99m pertechnetate (99mTc) or radioactive iodine (I^{131} or I^{123}) was the initial diagnostic procedure [13]. Benign nodules appear as hot nodules in scintigraphy images because they are more frequently hyperfunctioning and have a high captation rate of radionuclide, and physiologically, iodine [13]. By contrast, malignant nodules usually appear as cold in scintigraphy images because, frequently, they are not functioning [13]. However, this procedure is not as sensitive or specific as FNAB [13].

The diagnosis of papillary carcinoma is based on the nuclear morphology of the thyroid neoplasms [16]. By definition, PTC (Figure 3), has enlarged and elongated nuclei with crowding and overlap, irregular nuclear contour, chromatin clearing with peripheral margination of chromatin, giving rise to what has been described as *Orphan Annie Eye nuclei*; multiple micronucleoli located immediately underneath the nuclear membrane; nuclear grooves resulting from irregularity of nuclear contour; and intranuclear cytoplasmic pseudoinclusions from the accumulation of cytoplasm in prominent nuclear grooves [16]. Macroscopically, the lesions are firm and usually white in colour with an invasive appearance; lesional calcification is a common feature and cyst formation may be observed [17]. Microscopically, the neoplastic papillae contains a central core of fibrovascular tissue lined by one or several layers of cells with crowded oval nuclei [17].

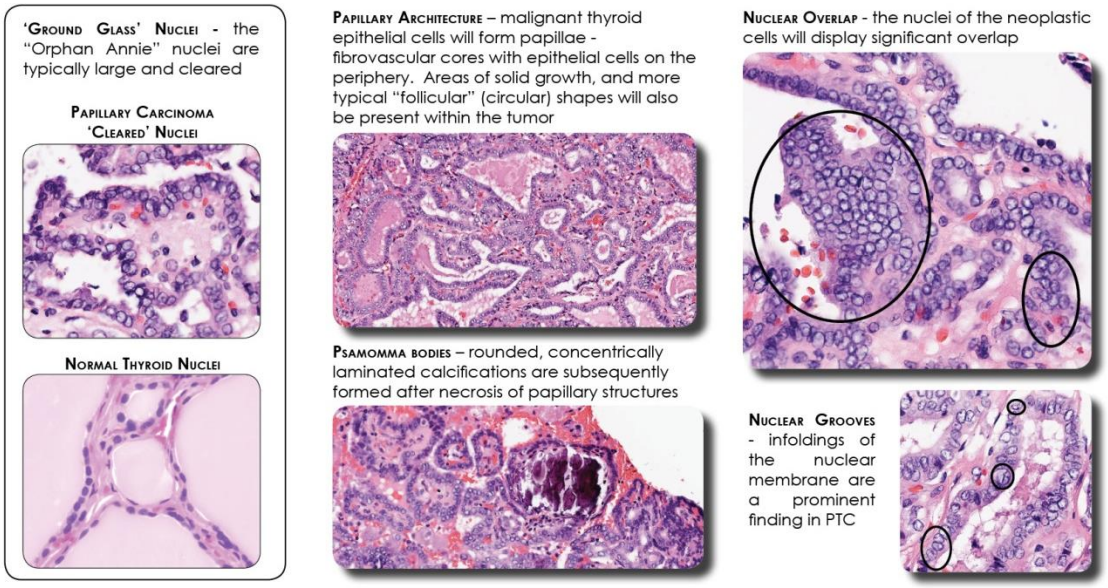

Figure 3 – Papillary thyroid carcinoma histologic features *(Schularick et al (2013) Pathology of papillary thyroid carcinoma, in Iowa Head and Neck protocols)*.

About 90% of the DTC are effectively cured by surgery (total or partial thyroidectomy) followed, when adequate, by RAI ablation therapy with 131 Iodine [8] (^{131}I is a β and γ emitting radionuclide and chemically identical to the non-radioactive form of iodine [7]). DTC of follicular origin retain, to varying degrees, the ability of normal thyrocytes to uptake and retain iodine [18]. The iodine trapping depends on the availability of NIS, which is an energy-dependent transport system regulated by the thyroid stimulating hormone (TSH) [7]. Since NIS is only present in thyroid follicular cells and DTCs, undifferentiated thyroid cancer and medullary thyroid cancer (parafollicular cell origin) are not responsive to RAI treatment [7]. In the absence of thyroid tissue (after surgical excision), serum thyroglobulin can be used as an excellent tumor marker for the detection of persistent or recurrent disease [19]. To achieve sufficient iodine uptake into tumor cells, RAI therapy requires high levels of TSH that can be achieved through thyroid hormone withdrawal or by injection of human recombinant TSH [18].

Contrarily to the majority of DTC that have a good prognosis, in 10% of the cases of DTC patients are diagnosed in an advanced stage of the disease, with local invasion and/or distant metastases and curing these cases with surgery and RAI therapy might be unlikely since these tumors tend to have a very low avidity for iodine [20]. The same happens with ATC and PDTC, whose tumoral cells are so dedifferentiated that they are no longer able to express NIS and uptake radioiodine; MTC, for biological reasons, a tumor derived from parafollicular C-cells, does not have ability to trap iodine [20]. For these cases, where there is not a rationale for using RAI therapy, there are other treatment

strategies. External beam radiation therapy (EBRT) and chemotherapy with cisplatin or doxorubicin can also be employed [13].

In the last decade, the increased understanding of the molecular mechanisms underlying thyroid tumors (see below) allowed the development of targeted therapies. The mitogen activated protein kinase (MAPK) pathway is one of the most studied pathways in the thyroid pathology and there are several therapies, approved or in clinical trials, that target this pathway [8]. Cabozantinib and Vandetanib are tyrosine kinase inhibitors (TKI) approved for treatment of MTC [21]. Sorafenib is also a TKI that targets BRAF, RET, VEGFR, PDGFRA and c-KIT, approved for treatment of late-stage metastatic DTC [8].

Papillary Thyroid Carcinoma histopathology and variants

There are several variants of PTC. The classic variant is characterized by complex papillae with thin fibrovascular cores. The papillae are covered by cuboidal and columnar cells with eosinophilic cytoplasm; psamomma bodies can also be identified and present a characteristic purple colour with laminated calcification [16].

The follicular variant of PTC presents a follicular architecture with nuclear features of papillary carcinoma. It is observed hypereosinophilia of colloid with peripheral scalloping, as well as, intrafollicular multinucleate giant cells and rare psamomma bodies [16]. Because these tumors can be confused with follicular adenomas and follicular carcinomas, the use of immunohistochemical and molecular markers are useful in the differential diagnosis [22].

The oncocytic variant is recognized by the presence of cells with abundant eosinophilic and granular cytoplasm as a consequence of mitochondrial accumulation. The cells can be arranged in classic papillae or in follicles, either with microfollicular ou macrofollicular architecture [16]. These tumors tend to have a distinct brown colour on gross examination [22].

The clear cell variant presents a papillary architecture and cytological features of PTC constituted by clear cells [22]. It is related to the oncocytic variant and tumors with clear cells usually have oncocytic cells as well [16].

The solid variant is characterized by unencapsulated and invasive borders. Cells are arranged in sheets intervened by fibrous stroma. Papillary formations are rare and the follicular pattern is partly maintained [16]. Vascular invasion and extrathyroidal extension are present in about a third of cases [22], that justifies the known aggressive behaviour of this variant, also associated with a high frequency of distant metastasis [16].

The tall cell variant is an uncommon and infiltrative tumor composed predominantly of cells whose length is at least 3 times their width. The cells usually have an abundant

eosinophilic cytoplasm and nuclear morphology that is typical of PTC [16]. These tumors tend to be larger than the classical PTC and necrosis, mitotic activity and extrathyroidal extensions are more frequently observed [22].

The columnar cell variant is a rare variant that is made up of pseudo stratified columnar cells. These cells may have supranuclear and subnuclear cytoplasmic vacuoles and some tumors may resemble endometrial or colonic adenocarcinomas [22]. This variant may be identical to the tall cell variant. The major difference is that tall cell variant has papillae that are delineated by a single layer of tall cells with an abundant eosinophilic cytoplasm and the presence of granules that give an oncocytic appearance [23].

The cribriform-morular variant is associated with familial adenomatous polyposis and Gardner Syndrome [22]. This variant is characterized by lobules of tumor separated by fibrous septa that have cribriform architecture characterized by rigid spaces in the lobules formed by arches of cells with no fibrovascular cores. Spindle cells and squamous morules can also be identified [16].

The diffuse sclerosing variant is more frequent in young adults [17]. It is characterized by diffuse involvement of the thyroid and both lobes are typically involved. Papillary structures in dilated lymphovascular spaces are often present; the tumor shows extensive squamous metaplasia, abundant psamomma bodies, stromal fibrosis and prominent lymphocytic thyroiditis [22].

Papillary microcarcinoma (mPTC) is defined as a papillary carcinoma that measures 1 cm or less in its maximum dimension [16]. These tumors are often found incidentally and are very common; the pattern of growth can be infiltrative or encapsulated; mPTC are frequently multiple (multifocal) [16]. mPTC incidence rates have been steadily increasing all over the world due to improvement of diagnostic methods [24]. In most of the cases, mPTC has a benign behaviour with an indolent course and excellent prognosis [24]. However, some have aggressive features and may require aggressive treatment [24]. The standard therapeutic approach for these tumors remains controversial. With the purpose of predicting those that will cause significant disease, many studies have been trying to associate poor prognostic markers with genetic alterations in this type of cancer.

EPIDEMIOLOGY

Thyroid carcinoma is the most frequent endocrine neoplasia and accounts for 1% of all malignancies, having an incidence higher in women than in men (3 times more frequent) [25].

The most frequent type of thyroid cancer is PTC (75-85%), followed by FTC (10-15%) and poorly differentiated/undifferentiated thyroid cancers (<5%) represent a minority of thyroid neoplasias [7, 26]. The overall 5 and 10-year survival rates of PTC are approximately 97% and 93%, respectively [7]. For FTC, the overall 5 and 10-year survival rates are 91% and 85%, respectively [7]. ATC is uncommon and very lethal. Its mean survival time is usually less than 6 months from the time of diagnosis and this outcome is not altered by current treatments [7]. MTC (~4%) is more aggressive than follicular cell derived thyroid carcinoma, being responsible for 8% to 15% of all thyroid cancer-related deaths and with a 10-year survival rate of 75-85% [27, 28]. Thyroid cancers can occur at any age but the risk peak for women is between their 40s and 50s and for men between their 60s and 70s [25].

According to RORENO (Registo Oncológico Regional do Norte), the incidence of thyroid cancer in the north of Portugal is 15 cases per 100 000 individuals and it is the third most prevalent cancer in women (8.2% of all cancers in women with 23.9 cases per 100 000 individuals) [29]. In Portugal, the overall mortality rate was 0,7 per 100 000 individuals [30]. The 5-year survival rate is 97.9% (women with slightly increase when compared with men), being the cancer with the best survival rate in Portugal [31].

Thyroid cancer incidence has continuously increased in the last three decades all over the world [32]. The increase is nearly exclusively due to increases in the incidence of the papillary histotype and small tumors rather than large tumors [32]. In spite of the steadily increased incidence, thyroid cancer mortality is reported stable at approximately 0.5 deaths per 100,000 persons [33]. Explanations for the worldwide increase of thyroid cancer incidence are controversial, with some experts believing that it is due to the increased diagnostic intensity [34]. In particular, the incidence rate in South Korea in 2011 was 15 times higher than in 1993 [35]. This increase resulted when providers added thyroid screening with ultrasonography to other cancer-screening tests paid by the government [36]. When the screening was stopped the incidence of thyroid cancer started to decreased. Overdiagnosis detects diseases that will not affect patient health and survival. Detecting these diseases not only will confer little benefit to the patient but may

also cause potential damage in terms of avoidable distress, possible adverse consequences of unnecessary treatment, and increasing economic cost [37]. Nevertheless, other experts believe that a true increase, due to environment and lifestyle changes, is also a possibility [32] [38].

Although the precise causes of thyroid cancer remain unclear, there are several observational studies that propose some factors that increase the risk of developing a thyroid cancer (TC), such as exposure to radiation, sex (women) and a diet low in iodine [25]. Radiation treatment of head and neck carcinomas and the exposure to radiation in Chernobyl accident have been two of the most studied risk factors for TC [39, 40]. In 2012, a meta-analysis of 7 cohort studies demonstrated the association of obesity and increased TC risk (by 18%) [41]. Diabetes mellitus have been also associated with increased TC risk [42-44]. Acromegaly, a rare syndrome that is caused by excess production of growth hormone, is highly associated with cancer risk, specifically TC [45-47]. Several studies propose that patients with benign thyroid diseases such as goiter, benign nodules, Hashimoto's thyroiditis (autoimmune disease) and hyperthyroidism are also at high risk of developing TC [48-50]. Genetic susceptibility is also associated with the risk of developing TC. Some studies demonstrated that individuals with family history of TC have an increased risk of developing TC [51, 52]. More controversially, some studies, unlike the majority of cancers, suggested that cigarette smoking and alcohol consumption were associated with decreased risk of thyroid cancer [53]. A meta-analysis of 31 observational studies also concluded that the risk of thyroid cancer was decreased 21% in ever-smokers compared to never-smokers [54].

GENETIC ALTERATIONS

PTC frequently has genetic alterations leading to the activation of the mitogen-activated protein kinase (MAPK) signalling pathway (Figure 4). Those include B-Raf Proto-Oncogene, Serine/Threonine Kinase (BRAF) and RAS Viral Oncogene Homolog (RAS) point mutations and *RET/PTC* rearrangements. Mutations involving one of these genes are found in >70% of PTC and they rarely overlap in the same tumor [55]. Recently, mutations in *telomerase reverse transcriptas*e (*TERT*) gene promoter were found in TC with an overall prevalence of near 10% [56] [57].

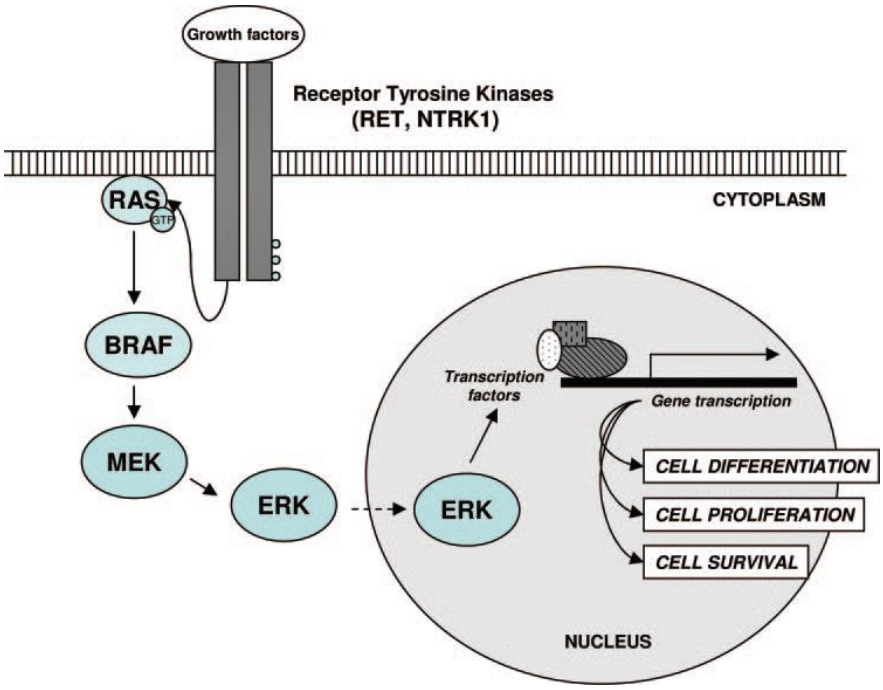

Figure 4 – Schematic representation of the MAPK signaling pathway. Physiologically, binding of growth factors to receptor TKs, results in receptor dimerization and activation via autophosphorylation of tyrosine residues in the intracellular domain. The activated receptor, through a series of adaptor proteins, leads to activation of RAS located at the inner face of the plasma membrane by substitution of GDP with GTP. The GTP-bound form of RAS binds to and recruits RAF proteins, mainly BRAF in thyroid follicular cells, to the plasma membrane. Activated BRAF is now able to phosphorylate and activate the MEK, which in turn phosphorylates and activates the ERK. Once activated, ERK phosphorylates cytoplasmic proteins and translocates into the nucleus, in which it regulates transcription of the genes involved in cell differentiation, proliferation, and survival. Alterations of this pathway in thyroid cancer can occur at different levels as a result of point mutations or rearrangement involving the RET, RAS and BRAF genes [2] *(Ciampi et al. (2007) RET/PTC Rearrangements and BRAF mutations in thyroid tumorigenesis, Endocrinology).*

BRAF point mutations

The *BRAF* gene on chromosome 7 (7q34) encodes the BRAF protein [58]. It belongs to the RAF protein family, being a serine-threonine protein kinase and a member of the RAS-RAF-MEK-ERK cell-signalling pathway (also known as the Mitogen Activated Protein Kinase (MAPK) pathway) [59]. This pathway regulates important cell functions including cellular growth, differentiation, proliferation, senescence and apoptosis [59]. BRAF phosphorylates and activates MEK, which in turn phosphorylates and activates ERK and all the downstream effector molecules of the MAPK pathway [60]. Once activated, ERKs can be translocated into the nucleus where they phosphorylate transcription factors, regulating their activity [60]. The most common genetic alterations in PTC refers to point mutations of *BRAF* [7]. More than 95% of *BRAF* mutations detected in TC are thymine to adenine conversions at position 1799 (T1799A) at exon 15, resulting in the substitution of valine by glutamate at residue 600 (V600E) [61]. This mutation causes constitutive activation of BRAF kinase and, thus, activation of the MAPK signalling

pathway, which is relevant for thyroid tumorigenesis [62]. In its wild-type conformation, residues G597 to V601 form a hydrophobic interaction with residues G465 to V472 in the ATP-binding site, keeping it inactivated [63]. The *BRAF* V600E mutation disrupts the hydrophobic interaction, enabling the BRAF kinase to fold into a catalytically active formation, resulting in an almost 500-fold increase in kinase activity [63]. According to a recent meta-analysis, *BRAF* V600E mutation has an overall prevalence of 57% in mPTCs, being the most common genetic alteration in this type of tumors [64]. In small percentages of PTC, K601E point mutations, small deletions or insertions around codon 600 and *AKAP9/BRAF* rearrangement can also be observed [7].

RAS point mutations

The *RAS* gene encodes a family of three highly homologous genes: Neuroblastoma RAS Viral Oncogene Homolog (NRAS), Harvey Rat Sarcoma Viral Oncogene Homolog (HRAS) and Kirsten Rat Sarcoma Viral Oncogene Homolog (KRAS). These 21-kDa membrane-associated proteins play a central role in the transduction of signals from tyrosine kinase and G protein-coupled receptors to effectors of the MAPK and PI3K-AKT signalling pathways, that is, from cell membrane receptors to their intracellular effector molecules. [65]. RAS proteins exist as an active form with guanosine triphosphatase (GTPase) activity and an inactive form that is bound to guanosine diphosphate (GDP) [7]. Point mutations produce oncogenic alleles of *RAS* that exhibit either increased affinity for GTP (mutations in codons 12 and 13) or inhibition of autocatalytic GTPase function (mutation in codon 61) [65]. Both mechanisms result in constitutive, aberrant activation of the downstream MAPK and PI3K/AKT signalling pathways [65]. Most series demonstrate predominance of *NRAS* 61 (Q61R; c.182A>G) mutant isoform in thyroid neoplasms [65]. There are also increasing evidence that RAS-positive PTC are mainly of the follicular variant subtype (FVPTC) [65]. According to a recent meta-analysis which evaluates this mutation in 106 tumors, the overall prevalence of RAS mutations in mPTCs is only 4% [64].

RET/PTC Rearrangements

Proto-oncogene *RET* (*rearranged during transfection*) encodes for a membrane-bound receptor tyrosine kinase and is highly expressed in calcitonin-producing parafollicular cells (C cells) in the thyroid gland [66]. *RET* gene is located on chromosome 10q11.2 [67] and contains three functional domains: an extracellular ligand-binding domain, a hydrophobic transmembrane domain and an intracellular tyrosine kinase domain [2]. In follicular cells it

can be activated by a chromosomal rearrangement know as *RET/PTC* rearrangement [68]. This rearrangement is characterized by a fusion of the 3' portion of *RET* gene to the 5' portion of several unrelated genes [2]. At least 12 different types of *RET/PTC* rearrangement have been identified [66]. The two most common are *RET/PTC1* (formed by the fusion with *H4* (*D10S170*) gene) and *RET/PTC3* (formed by the fusion with *NCOA4* (*ELE1*) gene) [66]. In *RET/PTC* rearrangements, fusion with protein partners possessing protein-protein interaction motifs provides RET/PTC kinases with dimerizing interfaces, which results in ligand-independent autophosphorylation of the tyrosine kinase domain [66]. The loss of the transmembrane domain in the rearranged protein results in the cytoplasmic expression of the constitutively active fusion gene. A recent meta-analysis reported an overall prevalence of 44% in mPTC, being the second most common genetic alteration in this type of tumors [64].

TERT promoter mutations

Telomeres are DNA-protein structures that protect chromosome ends which consist of arrays containing guanine-rich (G-rich) repeats $(TTAGGG)_n$ in vertebrates. These structures prevent the eventual loss of coding DNA due to the end replication problem, a limitation that causes telomere shortening within each cell division and leading, eventually, to cellular senescence or apoptosis [69]. Human adult somatic cells usually repress telomerase expression, although the enzyme continues to be expressed in proliferative cells (germ cells and tissue stem cells) [70].

Human *TERT* gene encodes the catalytic subunit of telomerase that together with a RNA component, TERC, maintains genomic integrity by telomere elongation [71]. Reactivation of telomerase is present in up to 90% of human cancers, and it allows proliferative cancer cells to maintain telomere length [70]. Mutations in the coding region of telomerase are very rare in human cancer [72]. In 2013, mutations in the promoter of the telomerase gene were reported for the first time, in melanoma [73, 74]. In the same year, *TERT* promoter mutations were reported in thyroid (follicular cell-derived tumors) with an overall prevalence of 10% [57]. A recent meta-analysis reported an overall prevalence of 4,6% in mPTC [64]. *TERT* promoter mutations occur in two hotspot positions, located -124 and -146 bp upstream from the ATG start site (-124 G>A and -146 G>A, C>T on the opposite strand) and confer enhanced *TERT* promoter activity, by putatively generating a novel consensus binding site (GGAA) for E-twenty six transformation-specific (ETS) transcription factors family within the *TERT* promoter region [57, 73, 74].

AIMS OF THE STUDY

AIMS OF THE STUDY

The general aim of this work was to evaluate the mPTC genetic profile. In order to do that, we intended to evaluate the prevalence of *BRAF* (exon 15, codon 600 region), *NRAS* (exon 3, codon 61 region) and *TERT* promoter mutations in a series of mPTC.

We also intended to investigate possible associations of those genetic alterations with classical clinicopathological features.

MATERIALS AND METHODS

MATERIALS AND METHODS

Samples

All the procedures described in this study were performed according to national ethical rules and with the approval of the ethic committee of Centro Hospitalar de São João (CHSJ).

Tumor samples were obtained from CHSJ between 1996 and 2013 and were stored as formalin-fixed paraffin-embedded (FFPE) tissue samples. Diagnosis and clinicopathological data were obtained by a pathologist and endocrinologist, both from CHSJ. This series included 113 patients and was selected according to outcome, specifically a worse outcome when compared to the majority of mPTC. Some cases are composed of more than one sample, as mPTC has frequently multifocality. Unfortunately, it was not possible to analyse all 113 patients due to lack of time.

The clinicopathological variables studied were gender, age, age at diagnosis, initial diagnosis, tumor size, histologic architecture variant, tumor localization in thyroid (left lobe, right lobe, isthmus, diffuse and pyramidal lobe), localization (central or peripheral), presence of thyroiditis (yes or no), vascular invasion (yes, suggestive or no), capsular invasion (yes or no), extrathyroidal invasion (yes, suggestive or no), presence of psammoma bodies (yes or no), lymph node metastasis (yes or no), multifocality (yes or no), tumor capsule (yes or no), tumor capsular invasion (yes or no), growth pattern (infiltrative, expansive or mixed), tumor margins (well defined or poorly defined), presence of necrosis (yes or no), intratumoral inflammatory infiltration (none, lymphocytic-scarce or lymphocytic), local metastasis (no or lymph node), number of lymph node metastasis, histologic relapse (yes or no) and years to relapse.

DNA extraction

Tumor areas were delimited, by a pathologist, in the haematoxylin and eosin (H&E) stained slides of each sample. The corresponding unstained slides were used for DNA extraction. The paraffin from FFPE slides was removed by immersion in xylene (Atom Scientific Ltd, United Kingdom) for 2 x 10 minutes and 2 x 5 minutes in ethanol 100% (Aga – Álcool e Géneros Alimentares, S.A., Portugal). Tumor areas, which were previously delimited by comparison with respective H&E slides, were macrodissected and transferred to a microcentrifuge tube. DNA was isolated using the GRS genomic DNA kit (GRiSP®, Portugal) and according to manufacturer's instructions. Finally, DNA was quantified by Nanodrop N-1000 Spectrophotometer (Thermo Scientific®, USA) and stored at 4 ºC.

Genetic alterations

Polymerase chain reaction (PCR) was performed using QIAGEN® Multiplex PCR kit in a final volume of 14 µl. This final mixture was composed of 6,25 µl of 2x QIAGEN PCR Master Mix (QIAGEN); 1,25 µl of Q-solution, 5x (QIAGEN); 3,5 µl of DNase and RNase free water; 0,25 µl each primer 100 uM (forward and reverse) and 2,5 µl of DNA template. PCR reaction was run in MyCycler™ thermal cycler (BIO RAD®, USA). The temperature conditions of PCR reaction consisted in an initial denaturation at 95ºC for 15 minutes, followed by 40 cycles with denaturation at 95ºC for 30 seconds, annealing of primers at 61ºC for 90 seconds and polymerase extension at 72ºC for 1 minute. After the 40 cycles the samples were subjected to a final extension at 72ºC for 10 minutes. In all PCR reactions it was used negative controls differing from the normal samples only by not having DNA template.

To confirm the efficiency of the PCR reaction, the products were run in a 2% agarose gel electrophoresis, using the SGTB 1x buffer (ref. GB01.0520, GRiSP, Portugal). The samples were mixed with 1µL of Loading Buffer with Gel Red® Nucleic Acid Gel Stain 3X (ref. 41003, Biotium, Inc, USA), where the loading buffer provides density to the sample and include colored dyes used to monitor the progress of the electrophoresis and the Gel Red® intercalate into the major grooves of the DNA and will be fluorescent under UV light, according to the manufacturer guidelines. To evaluate the size of the PCR products in the electrophoresis gel, 1kb Plus DNA Ladder (ref 10787-026, Invitrogen, USA) was used. The gel was analysed in ChemiDoc™ XRS Imaging System, BIORAD in an UV filter lamp (Model: Universal Hood II, Hercules, CA, USA - 50/60 Hz).

Before sequencing, all PCR products were purified to remove excess of primers, salts, dNTPs and enzymes from the PCR reaction, using the ExoSap method. ExoSap is composed by two hydrolytic enzymes, Exonuclease I 20U/µL (ref. #ENO582, Thermo Scientific) and Shrimp Alkaline Phosphatase (Fast AP Thermosensitive Alkaline Phosphatase 1U/µL, ref. #EF0651, Thermo Scientifics). For that purpose, it was added 1,5 µl of ExoSap to the PCR product, followed by incubation at 37ºC for 30 minutes, optimal temperature for the enzymes action, and 80 ºC for 15 minutes for their inactivation.

The PCR products were analysed by DNA sequencing (Sanger sequencing) using ABI Prism BigDye Terminator Kit v3.1 Cycle Sequencing (ref 4337455, Applied Biosystems®, United Kingdom). The final mixture had 2,5 µl of PCR product, 0,25 µl of BigDye, 3,4 µl of Sequencing buffer (Big Dye® Terminator v1.1, v1.3 5x sequencing buffer, ref 4336697, Applied Biosystems®), 0,3 µl of one of the primers (forward or reverse) and

3,4 µl of DNase and RNase free water. The final mixture was amplified in MyCycler™ thermal cycler (BIO RAD®) with an annealing temperature of 55ºC.

PCR sequencing products were purified using Zetadex-50 Superfine Gel Filtration Matrix (ref. TM-0104-E100.0-001, Biotech GmbH, Germany) columns to remove all ddNTPs not incorporated during sequencing reaction. After this step, it was added 15 µl of Hi-Di™ Formamide (ref 1403305, Applied Byosystems®) to the samples. Finally, samples were run in an ABI prism 3100 Genetic Analyzer (Perkin-Elmer, USA). Electropherograms of each sample were analysed with the Sequencing Analysis Software v5.4 (Applied Biosystems).

All positive cases for mutation were validated by a new independent analysis (this independent analysis was not performed as no positive cases for mutation were obtained).

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics version 24 (IBM, USA).

For the analysis of relationship between the continuous variables tumor size and age with clinicopathological characteristics, unpaired t-test and variance analysis was used. Chi-square test was used, with Fisher's correction when eligible, for analysis of relationship between the other clinicopathological characteristics. Frequency tables for each of the variables, expressed in frequency and percentage were also obtained. We considered statistically significant P-value<0.05.

RESULTS

RESULTS

This series included a total of 113 patients with the diagnosis of mPTC. A total of 66 DNA slides from 45 patients were analysed for molecular characterization.

It was our intention to classify molecularly these tumors, analysing the prevalence of *BRAF*, *NRAS* and *TERT* promoter mutations. However, only the analysis of *NRAS* mutations was successful.

Of the 45 patients, the mean age was 50, ranging from 21 to 77 years. The majority of the patients were older than 45 years (66.7%). There were a total of 3 male cases (6.7%), with a mean age of 57 (41-75) years, and 42 female cases (93.3%), with a mean age of 50 (21-77) years.

Of the 45 patients, 13 (28.9%) were incidental cases, which means that the microcarcinoma was not diagnosed *per se* but during pathologic examination of thyroid specimens after surgery for benign thyroid diseases or other malignant diseases (follicular thyroid tumor, papillary thyroid tumor and hurthle cell thyroid tumor).

In the 45 patients, 81 mPTCs were diagnosed. 21 patients (46.7%) had only 1 microcarcinoma and the remaining 24 (53.3%) patients had multifocality. Multifocality had the same number of unilateral and bilateral cases. Within the 24 cases with multifocality, 16 (66.7%) presented equal histotype architecture variant among all the microcarcinomas of the same case. Regarding the number of lesions in the cases presenting multifocality 18 patients (40.0%) had 2 microcarcinomas, 2 patients (4.4%) had 3 microcarcinomas, 2 patients (4.4%) 4 microcarcinomas and 2 (4.4%) patients presented 5 microcarcinomas. Tumors presented a maximum of 10 millimetres (mm) and a minimum of 1 mm in their maximum diameter, with a mean maximum diameter of 5.5 mm.

From the 81 mPTCs, 44 (54.3%) had follicular architecture, 25 (30.9%) classical architecture, 9 (11.1%) mixed architecture (half follicular and half classical) and 3 (3.7%) solid architecture – Table 1. 42 (51.9%) mPTCs were located in the right lobe, 38 (46.9%) in the left lobe and 1 (1.2%) in the pyramidal lobe.

Table 1 – Diagnosis of the architecture of the papillary thyroid microcarcinoma

Predominant architecture diagnosis	Frequency (n=81)	Percentage (%)
Follicular	44	54.3
Classical	25	30.9
Mixed	9	11.1
Solid	3	3.7

Of the 45 patients, 4 (8.9%) had tumor encapsulation and of those 4 all (100%) had invasion of the capsule. Furthermore, 21 (46.7%) patients had vascular invasion (cases with suggestive vascular invasion were included), 33 (73.3%) had extrathyroidal invasion (cases with suggestive extrathyroidal invasion were included) and 19 (42.2%) of the patients had tumor lymphocytic infiltrate (cases with scarce tumor lymphocytic infiltrate were included). The majority of the patients had an infiltrative tumor growth pattern (82.2%), poorly defined tumor margins (93.3%) and had no lymph node metastasis (91.1%). Only 2 patients (4.4%) had histologic relapse, which means that their relapse was confirmed by histologic analysis. 10 (22.2%) patients had Multinodular Goiter (MNG) and 16 (35.5%) colloid nodules. In 11 (24.4%) patients psammoma bodies were found – Table 2.

Table 2 – Pathological and molecular characteristics of the mPTCs in the 45 patients

Variables		Frequency (n=45)	Percentage (%)
Age	**<45 years**	15	33.3
	≥45 years	30	66.7
Gender	**Male**	3	6.7
	Female	42	93.3
Incidental mPTC		13	28.9
Multifocality	**No**	21	46.7
	Yes	24	53.3
Multifocality side	**Unilateral**	12	50.0
	Bilateral	12	50.0
MNG		10	22.2
Colloid nodules		16	35.5
Psammoma bodies		11	24.4
Tumor capsule		4	8.9
Tumor capsule invasion		4	8.9

Vascular invasion (cases with suggestive vascular invasion were included)		21	46.7
Extrathyroidal invasion (cases with suggestive extrathyroidal invasion were included)		33	73.3
Lymphocytic infiltrate		19	42.2
Tumor margins	**Well-defined**	3	6.7
	Poorly-defined	42	93.3
Growth pattern	**infiltrative**	37	82.2
	expansive	3	6.7
	mixed	5	11.1
Lymph node metastasis		4	8.9
Histologic relapse		2	4.4
***NRAS* mutation**		0	0

We also did a comparative analysis between the 2 most prevalent mPTC architecture variants present in this series, classical (c-mPTC) and follicular (fv-mPTC), to see the distribution of the clinicopathological characteristics in those variants.– Table 3.

Of the 45 patients, 13 were diagnosed with c-mPTC (single or multiple) with a mean age of 47 years and 20 with fv-mPTC (single or multiple) with a mean age of 51 years.

Table 3 – Pathological and molecular characteristics of c-mPTC and fv-mPTC.

Variables		**c-mPTC (n=13)**	**fv-mPTC (n=20)**
Age (%)	**<45 years**	4 (30.8)	8 (40.0)

	≥45 years	9 (69.2)	12 (60.0)
Gender (%)	**Male**	1 (7.7)	2 (10.0)
	Female	12 (92.3)	18 (90.0)
Incidental mPTC (%)		3 (23.1)	8 (40.0)
Multifocality (%)	**No**	8 (61.5)	10 (50.0)
	Yes	5 (38.5)	10 (50.0)
Tumor capsule (%)		1 (7.7)	1 (5.0)
Tumor capsule invasion (%)		1 (7.7)	1 (5.0)
Vascular invasion (cases with suggestive vascular invasion were included) **(%)**		7 (53.9)	9 (45)
Extrathyroidal invasion (cases with suggestive extrathyroidal invasion were included) **(%)**		6 (46.2)	17 (85.0)
Lymphocytic infiltrate (cases with scarce tumor lymphocytic infiltrate were included) **(%)**		6 (46.2)	11 (55.0)
Tumor margins (%)	**Well-defined**	1 (7.7)	1 (5.0)
	Poorly-defined	12 (92.3)	19 (95.0)
Growth pattern (%)	**infiltrative**	7 (53.8)	19 (95.0)
	expansive	2 (15.4)	1 (5)

	mixed	4 (30.8)	0 (0)
Lymph node metastasis (%)		2 (15.4)	1 (5)
Histologic relapse (%)		1 (7.7)	0 (0)
***NRAS* mutation (%)**		0 (0)	0 (0)

When the two most prevalent variants were compared, c-mPTC and fv-mPTC, statistical significant differences were observed. The c-mPTC group had more psammoma bodies (53.8% vs 15.0%) and less extrathyroidal invasion (41.7% vs 85.0%) when compared with the fv-mPTC group – Table 4.

Table 4 – Comparison between the classical mPTC and follicular variant mPTC, in relation to the variables extrathyroidal invasion and psammoma bodies.

Variables		**Variant diagnosis**		**p-value**
		c-mPTC	**fv-mPTC**	
Extrathyroidal invasion (%)	**Yes**	**5** (41.7)	**17** (85.0)	**0.018**
	No	**7** (58.3)	**3** (15.0)	
Psammmoma bodies (%)	**Yes**	**7** (53.8)	**3** (15.0)	**0.026**
	No	**6** (46.2)	**17** (85.0)	

When the incidental cases of mPTC were compared with the non-incidental cases, it was possible to report statistical significant differences with the incidental cases being more frequentely associated with colloid nodules (76.9% vs 18.8%) and with multinodular goiter (MNG) (61.5% vs 6.3%) when compared with the non-incidental cases of mPTC. It is also possible to report a larger mean maximum diameter (mean of all tumors of each case) in the group of the non-incidental cases – Table 5.

Table 5 – Comparison between the incidental and non-incidental cases of mPTC, in relation to the variables colloid nodules, multinodular goiter (MNG) and mean maximum diameter of all tumors of the case.

Variables		Incidental mPTC		p-value
		Yes	No	
Colloid nodules (%)	Yes	**10** (76.9)	**6** (18.8)	**0.000**
	No	**3** (23.1)	**26** (81.3)	
MNG (%)	Yes	**8** (61.5)	**2** (6.3)	**0.000**
	No	**5** (38.5)	**30** (93.8)	
Mean tumor size (mm)		**5.12**	**6.57**	**0.033**

When the cases with and without multifocality were compared, it was possible to report a tendency, with the cases with multifocality being more frequent in females (100% vs 85.7%), having less lymph node metastasis (0% vs 19.0%) and the tumor margins being frequently poorly-defined (100% vs 85.7%) when compared with the cases without multifocality – Table 6.

Table 6 – Comparison between the cases with and without multifocality, in relation to the variables gender, lymph node metastasis and tumor margins

Variables		Multifocality		p-value
		Yes	No	
Gender (%)	Male	**0** (0)	**3** (14.3)	**0.094**
	Female	**24** (100)	**18** (85.7)	
Lymph node metastasis (%)	Yes	**0** (0)	**4** (19.0)	**0.040**
	No	**24** (100)	**17** (81.0)	
Tumor margins (%)	Well-defined	**0** (0)	**3** (14.3)	**0.094**
	Poorly-defined	**24** (100)	**18** (85.7)	

When the group with and without lymph node metastasis were compared, it was possible to report statistical significant differences. The group with lymph node metastasis had more psammoma bodies (75.0% vs 19.5%) and less multifocality (0% vs 58.5%) when compared with the group without lymph node metastasis – Table 7.

Table 7 – Comparison between the group with and without lymph node metastasis, in relation to the variables psammoma bodies and multifocality

Variables		Lymph node metastasis		p-value
		Yes	No	
Psammoma bodies (%)	Yes	**3** (75.0)	**8** (19.5)	**0.040**
	No	**1** (25.0)	**33** (80.5)	
Multifocality (%)	Yes	**0** (0)	**24** (58.5)	**0.040**
	No	**4** (100)	**17** (41.5)	

When the group with and without tumor capsule were compared, it was possible to report some statistical significant differences, with the group with tumor capsule having all invasion of the tumor capsule (100 % vs 0%) and more well-defined tumor margins (50.0 % vs 2.4%) when compared with the group with no tumor capsule. However, the group with tumor capsule had less poorly-defined tumor margins (50.0% vs 97.6%) – Table 8.

Table 8 – Comparison between the group with and without tumor capsule, in relation to the variables Tumor capsule invasion and tumor margins.

Variables		Tumor capsule		p-value
		Yes	No	
Tumor capsule invasion (%)	Yes	**4** (100)		**0.000**
	No	**0** (0)		
Tumor	Well-	**2**	**1**	**0.018**

margins (%)	**defined**	(50)	(2.4)	
	Poorly-defined	**2** (50)	**40** (97.6)	

When the group with tumors with a maximum diameter of less than 5 mm was compared with the group with at least one tumor with 5 mm or more, it was possible to report a tendency, although the difference is not statistically significant. 75.0% of the cases belonging to the group with at least one tumor with 5 mm or more are non-incidental cases and 60.0% of the cases with a maximum diameter inferior to 5 mm are incidental cases – Table 9.

Table 9 – Comparison between the group with tumors with maximum diameter inferior to 5 mm and the group with at least one tumor with maximum diameter of 5 mm or more, in relation to the variable Incidental mPTC.

Variables		**Tumor size**		**p-value**
		<5	**≥5**	
Incidental mPTC (%)	**Yes**	**3** (60.0)	**10** (25.0)	**0.136**
	No	**2** (40.0)	**30** (75.0)	

The 66 genotyped samples, from the 45 patients, analysed for the *NRAS* mutations were all (100%) wild-type, which included 63 tumor samples and 3 lymph node metastasis - Figure 5 and 6.

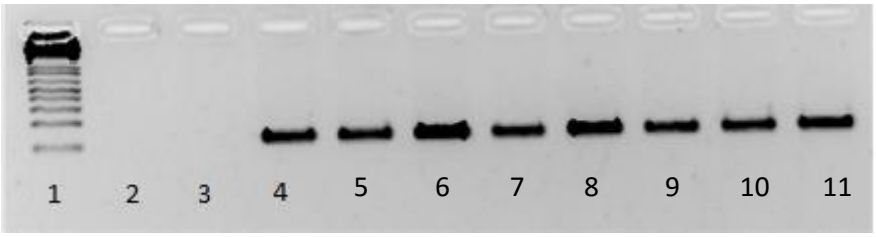

Figure 5 – Representative results of an agaroses gel electrophoresis for the PCR of NRAS codon 61 region, with a size of about 119 basepairs (1-DNA ladder; 2 & 3-negative control; 5 to 11-Positive case for amplification of NRAS.

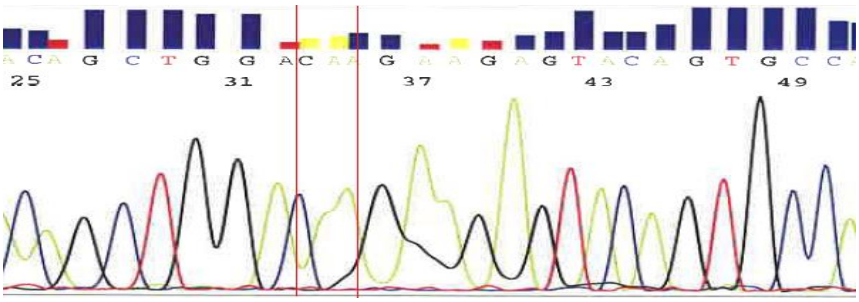

Figure 6 – Wild-type representative results obtained through sequencing analysis of NRAS codon 61 (bounded by 2 red lines) region, primer forward.

DISCUSSION

DISCUSSION

Papillary thyroid microcarcinoma (mPTC) constitutes a topic of intense debate in endocrine oncology, as these cancers have a spectrum of behaviour that ranges from incidentally detected and clinically indolent tumours, a minority that behave more aggressively and a few that can become virtually untreatable neoplasms [24]. The importance of genetic characterization of mPTC is to obtain tools that can predict those that will cause significant disease. In this work we intend to evaluate the genetic profile of a series of mPTC from the North of Portugal, evaluating the prevalence of BRAF V600E, RAS and TERT promoter mutations. We also evaluated a number of clinicopathological features.

In this study we have access to a series of mPTC that were selected based in the behaviour of the disease. The series is mainly composed of “worse” prognosis cases i.e. cases that, at presentation or in the follow-up period, showed vascular invasion, extrathyroidal invasion, metastasis or histologic relapse.

We will start with the analysis of the clinicopathological characteristics of the 45 mPTC cases included in in our study [24].

In this work, the female cases represented 93.3% of all cases. A recent meta-analysis, addressing 75 studies, estimates that 82% of mPTC belong to women [24]. The reason(s) underlying the higher incidence of mPTCs and PTCs in women remains not explained. Some authors advanced the fact that estrogen is a potent growth factor for malignant thyroid cells [75]. In fact, it was demonstrated that estrogen has the ability to activate MAPK and PI3K signalling pathways, two pathways with major roles in thyroid oncogenesis [75-78].

Multifocality is a common feature of mPTC and was also observed in this work. According to a recently published meta-analysis (2016) addressing 57 studies, multifocality prevalence in mPTCs is 28.0%. The multifocality prevalence of the series in this work was 53.0%. There are some studies that correlate the presence of multifocality with worse clinicopathological features, and that can be a possible explanation for the higher incidence of multifocality in this series. In fact, a recent work states that multifocality is an important factor for predicting tumor recurrence in mPTC [79]. Another study reports a higher capsular invasion, extrathyroidal invasion and lymph node metastasis in multifocal PTCs [80]. Interestingly, it was also reported an increase of these worse clinicopathological features in multifocal tumors that have a tumor diameter of less

than 10 mm, mPTCs, comparing with unifocal tumors with more than 10 mm [80]. Contrary to what a recent meta-analysis reports, in our results lymph node metastasis was more prevalent in unifocal tumors, with all four lymph node metastasis cases belonging to cases without multifocality [81]. Obviously, the numbers are too scarce to allow any meaningful tendency.

mPTC are tumors frequently found during histopathological examination of the thyroid glands removed during necropsy or surgery for nonmalignant thyroid disease, which makes them incidental tumors [24]. A meta-analysis of 25 studies reports that 57% of mPTC are diagnosed incidentally. [24]. The fact that the mPTC series used in this work had only 28.9% of incidental cases can be associated to the fact that this is a series with aggressive features. Probably, most of the patients of this series had some symptoms and their mPTCs were diagnosed on clinical grounds, being non-incidental. This is confirmed by the observation that in the non-incidental cases 81.3% did not present colloid nodules and 93.8% did not have MNG (Table 5). It is also interesting to see that the non-incidental cases have a maximum diameter superior to the incidental cases, which is in accordance to the findings of a recent work that compared incidental with non-incidental mPTC and observed larger tumors in the non-incidental group [82].

When we compare cases with and without lymph node metastasis, it is interesting to see that 75.0% of the cases with lymph node metastasis had psammoma bodies and that 80.5% of the cases with no lymph node metastasis did not present psammoma bodies, showing that psamomma bodies are almost exclusive of cases with lymph node metastasis. A study that analysed 258 PTCs found that the presence of psammoma bodies was significantly correlated with lymph node metastasis [83]. It is also interesting to see that classical variant presents more frequently psammoma bodies (table 4). There is some evidence that psammoma bodies are much more prevalent in classical mPTC [23].

When we compare the prevalence of the different mPTC variants it is possible to report a higher prevalence of the follicular variant, 54.3%, comparing with 30.9% of the classical variant and a minority of the other two variants. The same meta-analysis reported before, accounts that classical variant is the most prevalent, with 71.0% and follicular variant the second most prevalent with 19.0% [24]. A recent study of 1990 cases that evaluated the outcome of mPTC, found an association between lymph node metastasis and follicular variant [84]. This finding suggests that this variant might be more aggressive and that can be a possible explanation for the higher prevalence of follicular variant in the series used in this work. The hypothesis that follicular variant is more aggressive was supported by the higher prevalence of extrathyroidal invasion in this variant when compared with classical variant (table 4).

The series included a total of 113 patients with the diagnosis of mPTC. Unfortunately, it was not possible to do the molecular analysis of all of them in time, as the molecular characterization was dependent on the analysed slides sent by the pathologist and the pathological features were also dependent on the pathologist's analysis. For this reason only 45 cases were available for the molecular analysis.

Although it was initially planned to analyse BRAF (exon 15, codon 600 region), TERT promoter and NRAS (exon 3, codon 61 region) mutations, only NRAS mutation analysis was successfully performed

The *TERT* promoter sequence proved to be very difficult to amplify, as it is a gene promoter and it is known its high GC rich content. We failed to molecularly characterize *TERT* promoter in time for inclusion in this thesis. Since this sequence has successfully been genotyped in the past, in the research group were this work took place, it made us question about possible explanations for this problem. Due to the small size of microcarcinomas, the amount of DNA extracted from mPTC tissues was reduced, that could in part explain the increased difficulty in genotyping this sequence, although, previous mPTC have been studied in the group with similar amounts of material. Only two published studies report the analysis of TERT promoter mutations in mPTC, with an overall prevalence of 4.6%, ranging from 0%[57] to 4.7%[85]. One of these studies searched for associations between this mutation and unfavorable clinical features and nothing was found to be correlated [85]. This contrasts with the association found between TERT promoter mutations and worse outcome and disease-specific mortality in PTC [86].

Contrarily to *TERT* genotyping, *BRAF* amplification was successfully obtained. However, these results failed to be validated in a reliable way and therefore this data could not be used in order to not introduce biased or false results. BRAF V600E mutation is the most studied genetic alteration in mPTC and according to a recent-meta-analysis it has an overall prevalence of 57% (ranging from 0% to 90.7%) [64]. This prevalence is very similar to the 51% (ranging from 27.3% to 87.1%), reported in another recent meta-analysis of 30 studies on PTC [87]. Many studies found association between BRAF mutation and some clinicopathological features predictive of tumor aggressiveness [85, 88-102].

The fact that the sequence amplified of *NRAS* presented about 119 base pairs (bp) and the other two, *BRAF* and *TERT* promoter, have near 200 base pairs might explain the efficiency of this amplification, as a smaller sequence is more easily amplified. So, we did not face major complications in genotyping *NRAS*.

In this work, the prevalence of NRAS was 0% in the tumors and lymph node metastasis analysed. So far, there are only 4 published studies reporting the analysis of *RAS* mutations in mPTCs, with an overall prevalence of 3,8% (4/106), ranging from 0 to

5% [64, 103-106]. Interestingly, one of the studies that found *RAS* mutations stratified the series in two groups and reported no *RAS* mutations in the aggressive group, with all mutations belonging to the non-aggressive group [104]. This is an interesting result, as the analysis presented in this thesis was also done in an aggressive mPTC series and raises the question if aggressive mPTCs could have a lower frequency of *RAS* mutations. However, the absence of gene mutations precluded the search for associations of *RAS* with clinicopathological features of the mPTC. The general low prevalence of this mutation in mPTC contrasts with the observed frequency in normal PTCs that usually tend to affect 10% of the cases studied, with follicular variants presenting an higher incidence [7].

Genetic alterations of mPTC and the study of their association with clinicopathological features are of major importance to predict those cases that will cause significant disease. This series is composed by aggressive cases of mPTC. Their genetic characterization, when finished, will be an important tool for better understanding these uncommon cases with aggressive behavior.

CONCLUSIONS AND FUTURE PERSPECTIVES

CONCLUSIONS AND FUTURE PERSPECTIVES

Although the cases present in this series were selected based on their aggressive features, we confirmed the indolent clinical course of this type of tumor, with only 8.9% of lymph node metastasis and no distant metastasis or disease-specific mortality. It was also possible to see a high number of multifocal, incidental and female cases, as expected. Interestingly, follicular architecture was predominant in this series and that can be related with the aggressive features of these cases. Furthermore, the genetic characterization of this series revealed no mutations in NRAS gene. The work presented on this thesis is still part of an ongoing work with more cases to be analysed for genetic alterations in *BRAF, NRAS* and *TERT* promoter. The results arising from this future analysis are of major importance for better understanding the relationship between genetic alterations and clinical outcome in this type of tumors. It remains to be found a marker that could distinguish which of these mPTC will indeed present a worse prognosis and will require a more radical approach in order to treat them. Additional, as observed in some countries, the increased detection of these small lesions represents an economic burden. Therefore, we aim to identify those that will cause significant disease so an aggressive therapeutic approach can be taken.

BIBLIOGRAPHIC REFERENCES

1. Rousset, B.A., *How Iodide Reaches its Site of Utilisation in the Thyroid Gland Involvement of Solute Carrier 26A4 (Pendrin) and Solute Carrier 5A8 (Apical Iodide Transporter).* European Endocrinology, 2007. **00**(1): p. 81.
2. Ciampi, R. and Y.E. Nikiforov, *RET/PTC rearrangements and BRAF mutations in thyroid tumorigenesis.* Endocrinology, 2007. **148**(3): p. 936-41.
3. Kaplan, E., et al., *Chapter 21 SURGERY OF THE THYROID*, in *Endotext*, L.J. De Groot, et al., Editors. 2000: South Dartmouth (MA).
4. Nussey, S. and S. Whitehead, in *Endocrinology: An Integrated Approach*. 2001: Oxford.
5. Dumont, J., et al., *Ontogeny, Anatomy, Metabolism and Physiology of the Thyroid*, in *Endotext*, L.J. De Groot, et al., Editors. 2000: South Dartmouth (MA).
6. Johansson, E., et al., *Revising the embryonic origin of thyroid C cells in mice and humans.* Development, 2015. **142**(20): p. 3519-28.
7. Omur, O. and Y. Baran, *An update on molecular biology of thyroid cancers.* Crit Rev Oncol Hematol, 2014. **90**(3): p. 233-52.
8. Perri, F., et al., *Targeted therapy: a new hope for thyroid carcinomas.* Crit Rev Oncol Hematol, 2015. **94**(1): p. 55-63.
9. Pacini, F., et al., *Thyroid cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up.* Ann Oncol, 2012. **23 Suppl 7**: p. vii110-9.
10. Neff, R.L., et al., *Anaplastic thyroid cancer.* Endocrinol Metab Clin North Am, 2008. **37**(2): p. 525-38, xi.
11. Wells, S.A., Jr., et al., *Revised American Thyroid Association guidelines for the management of medullary thyroid carcinoma.* Thyroid, 2015. **25**(6): p. 567-610.
12. Cobin, R.H., et al., *AACE/AAES medical/surgical guidelines for clinical practice: management of thyroid carcinoma. American Association of Clinical Endocrinologists. American College of Endocrinology.* Endocr Pract, 2001. **7**(3): p. 202-20.
13. Baldwin, K.M. *Papillary Thyroid Carcinoma*. 2015 [cited 2015; Available from: http://emedicine.medscape.com/article/282276-overview.
14. Kessler, A., et al., *Accuracy and consistency of fine-needle aspiration biopsy in the diagnosis and management of solitary thyroid nodules.* Isr Med Assoc J, 2005. **7**(6): p. 371-3.
15. Dean, D.S. and H. Gharib, *Fine-Needle Aspiration Biopsy of the Thyroid Gland*, in *Endotext*, L.J. De Groot, et al., Editors. 2000: South Dartmouth (MA).
16. Al-Brahim, N. and S.L. Asa, *Papillary thyroid carcinoma: an overview.* Arch Pathol Lab Med, 2006. **130**(7): p. 1057-62.
17. LiVolsi, V.A., *Papillary thyroid carcinoma: an update.* Mod Pathol, 2011. **24 Suppl 2**: p. S1-9.
18. Grewal, R.K., A. Ho, and H. Schoder, *Novel Approaches to Thyroid Cancer Treatment and Response Assessment.* Semin Nucl Med, 2016. **46**(2): p. 109-18.
19. Bal, C.S. and A.K. Padhy, *Radioiodine Remnant Ablation: A Critical Review.* World J Nucl Med, 2015. **14**(3): p. 144-55.
20. Viola, D., et al., *Treatment of advanced thyroid cancer with targeted therapies: ten years of experience.* Endocr Relat Cancer, 2016. **23**(4): p. R185-205.
21. Shaw, A.T., et al., *Tyrosine kinase gene rearrangements in epithelial malignancies.* Nat Rev Cancer, 2013. **13**(11): p. 772-87.
22. Lloyd, R.V., D. Buehler, and E. Khanafshar, *Papillary thyroid carcinoma variants.* Head Neck Pathol, 2011. **5**(1): p. 51-6.

23. Gonzalez-Gonzalez, R., et al., *Papillary thyroid carcinoma: differential diagnosis and prognostic values of its different variants: review of the literature.* ISRN Oncol, 2011. **2011**: p. 915925.
24. Dideban, S., et al., *Thyroid Papillary Microcarcinoma: Etiology, Clinical Manifestations,Diagnosis, Follow-up, Histopathology and Prognosis.* Iran J Pathol, 2016. **11**(1): p. 1-19.
25. Society, A.C. *What are the risk factors for thyroid cancer?* ; Available from: http://www.cancer.org/cancer/thyroidcancer/detailedguide/thyroid-cancer-risk-factors.
26. Cabanillas, M.E. and M.A. Habra, *Lenvatinib: Role in thyroid cancer and other solid tumors.* Cancer Treat Rev, 2016. **42**: p. 47-55.
27. Pusztaszeri, M.P., M. Bongiovanni, and W.C. Faquin, *Update on the cytologic and molecular features of medullary thyroid carcinoma.* Adv Anat Pathol, 2014. **21**(1): p. 26-35.
28. Sippel, R.S., M. Kunnimalaiyaan, and H. Chen, *Current management of medullary thyroid cancer.* Oncologist, 2008. **13**(5): p. 539-47.
29. RORENO, *Registo Oncológico Regional do Norte 2010*. Instituto Portugûes de Oncologia do Porto: Porto.
30. RORENO, *Registo Oncológico Nacional 2010*. Porto.
31. RORENO, *Sobrevivência Global 2007-2008*. Instituto Português de Oncologia do Porto.
32. Pellegriti, G., et al., *Worldwide increasing incidence of thyroid cancer: update on epidemiology and risk factors.* J Cancer Epidemiol, 2013. **2013**: p. 965212.
33. Davies, L. and H.G. Welch, *Increasing incidence of thyroid cancer in the United States, 1973-2002.* JAMA, 2006. **295**(18): p. 2164-7.
34. Grodski, S., et al., *Increasing incidence of thyroid cancer is due to increased pathologic detection.* Surgery, 2008. **144**(6): p. 1038-43; discussion 1043.
35. Ahn, H.S., H.J. Kim, and H.G. Welch, *Korea's thyroid-cancer "epidemic"--screening and overdiagnosis.* N Engl J Med, 2014. **371**(19): p. 1765-7.
36. Ahn, H.S. and H.G. Welch, *South Korea's Thyroid-Cancer "Epidemic"--Turning the Tide.* N Engl J Med, 2015. **373**(24): p. 2389-90.
37. Schlumberger, M., et al., *Treatment and follow-up of low-risk patients with thyroid cancer.* Nat Rev Endocrinol, 2011. **7**(10): p. 625-8.
38. Rego-Iraeta, A., et al., *Time trends for thyroid cancer in northwestern Spain: true rise in the incidence of micro and larger forms of papillary thyroid carcinoma.* Thyroid, 2009. **19**(4): p. 333-40.
39. Giannoula, E., A. Gkantaifi, and I. Iakovou, *[Radiation treatment of head and neck carcinomas as a risk factor for thyroid carcinomas].* Hell J Nucl Med, 2016. **19**(1): p. 65-74.
40. LiVolsi, V.A., et al., *The Chernobyl thyroid cancer experience: pathology.* Clin Oncol (R Coll Radiol), 2011. **23**(4): p. 261-7.
41. Zhao, Z.G., et al., *Overweight, obesity and thyroid cancer risk: a meta-analysis of cohort studies.* J Int Med Res, 2012. **40**(6): p. 2041-50.
42. Yeo, Y., et al., *Diabetes mellitus and risk of thyroid cancer: a meta-analysis.* PLoS One, 2014. **9**(6): p. e98135.
43. Schmid, D., et al., *Physical activity, diabetes, and risk of thyroid cancer: a systematic review and meta-analysis.* Eur J Epidemiol, 2013. **28**(12): p. 945-58.
44. Oberman, B., et al., *Relationship between obesity, diabetes and the risk of thyroid cancer.* Am J Otolaryngol, 2015. **36**(4): p. 535-41.
45. Dagdelen, S., N. Cinar, and T. Erbas, *Increased thyroid cancer risk in acromegaly.* Pituitary, 2014. **17**(4): p. 299-306.
46. dos Santos, M.C., et al., *Thyroid cancer in patients with acromegaly: a case-control study.* Pituitary, 2013. **16**(1): p. 109-14.
47. Uchoa, H.B., et al., *Prevalence of thyroid diseases in patients with acromegaly: experience of a Brazilian center.* Arq Bras Endocrinol Metabol, 2013. **57**(9): p. 685-90.

48. Yeh, N.C., et al., *Hyperthyroidism and thyroid cancer risk: a population-based cohort study.* Exp Clin Endocrinol Diabetes, 2013. **121**(7): p. 402-6.
49. Tam, A.A., et al., *Thyroid nodules and thyroid cancer in Graves' disease.* Arq Bras Endocrinol Metabol, 2014. **58**(9): p. 933-8.
50. Noureldine, S.I. and R.P. Tufano, *Association of Hashimoto's thyroiditis and thyroid cancer.* Curr Opin Oncol, 2015. **27**(1): p. 21-5.
51. Mazeh, H., et al., *In patients with thyroid cancer of follicular cell origin, a family history of nonmedullary thyroid cancer in one first-degree relative is associated with more aggressive disease.* Thyroid, 2012. **22**(1): p. 3-8.
52. Myung, S.K., et al., *Risk Factors for Thyroid Cancer: a Hospital-Based Case-Control Study in Korean Adults.* Cancer Res Treat, 2016.
53. Kitahara, C.M., et al., *Cigarette smoking, alcohol intake, and thyroid cancer risk: a pooled analysis of five prospective studies in the United States.* Cancer Causes Control, 2012. **23**(10): p. 1615-24.
54. Cho, Y.A. and J. Kim, *Thyroid cancer risk and smoking status: a meta-analysis.* Cancer Causes Control, 2014. **25**(9): p. 1187-95.
55. Soares, P., et al., *BRAF mutations and RET/PTC rearrangements are alternative events in the etiopathogenesis of PTC.* Oncogene, 2003. **22**(29): p. 4578-80.
56. Liu, X., et al., *Highly prevalent TERT promoter mutations in aggressive thyroid cancers.* Endocr Relat Cancer, 2013. **20**(4): p. 603-10.
57. Vinagre, J., et al., *Frequency of TERT promoter mutations in human cancers.* Nat Commun, 2013. **4**: p. 2185.
58. Hussain, M.R., et al., *BRAF gene: From human cancers to developmental syndromes.* Saudi J Biol Sci, 2015. **22**(4): p. 359-73.
59. Caronia, L.M., J.E. Phay, and M.H. Shah, *Role of BRAF in thyroid oncogenesis.* Clin Cancer Res, 2011. **17**(24): p. 7511-7.
60. Peyssonnaux, C. and A. Eychene, *The Raf/MEK/ERK pathway: new concepts of activation.* Biol Cell, 2001. **93**(1-2): p. 53-62.
61. Xing, M., *Molecular pathogenesis and mechanisms of thyroid cancer.* Nat Rev Cancer, 2013. **13**(3): p. 184-99.
62. Nikiforov, Y.E., *Thyroid carcinoma: molecular pathways and therapeutic targets.* Mod Pathol, 2008. **21 Suppl 2**: p. S37-43.
63. Wan, P.T., et al., *Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF.* Cell, 2004. **116**(6): p. 855-67.
64. Cunha Rodrigues, A.P., Gustavo; Sobrinho-Simões, Manuel; Soares, Paula *The genetics of papillary microcarcinomas of the thyroid: diagnostic and prognostic implications.* Current Genomics (in press), 2016.
65. Howell, G.M., S.P. Hodak, and L. Yip, *RAS mutations in thyroid cancer.* Oncologist, 2013. **18**(8): p. 926-32.
66. Antonelli, A., et al., *RET TKI: potential role in thyroid cancers.* Curr Oncol Rep, 2012. **14**(2): p. 97-104.
67. Pasini, B., et al., *The physical map of the human RET proto-oncogene.* Oncogene, 1995. **11**(9): p. 1737-43.
68. de Groot, J.W., et al., *RET as a diagnostic and therapeutic target in sporadic and hereditary endocrine tumors.* Endocr Rev, 2006. **27**(5): p. 535-60.
69. Harley, C.B., A.B. Futcher, and C.W. Greider, *Telomeres shorten during ageing of human fibroblasts.* Nature, 1990. **345**(6274): p. 458-60.
70. Kim, N.W., et al., *Specific association of human telomerase activity with immortal cells and cancer.* Science, 1994. **266**(5193): p. 2011-5.
71. Heidenreich, B., et al., *TERT promoter mutations in cancer development.* Curr Opin Genet Dev, 2014. **24**: p. 30-7.

72. Vinagre, J., et al., *Telomerase promoter mutations in cancer: an emerging molecular biomarker?* Virchows Arch, 2014. **465**(2): p. 119-33.
73. Horn, S., et al., *TERT promoter mutations in familial and sporadic melanoma.* Science, 2013. **339**(6122): p. 959-61.
74. Huang, F.W., et al., *Highly recurrent TERT promoter mutations in human melanoma.* Science, 2013. **339**(6122): p. 957-9.
75. Derwahl, M. and D. Nicula, *Estrogen and its role in thyroid cancer.* Endocr Relat Cancer, 2014. **21**(5): p. T273-83.
76. Manole, D., et al., *Estrogen promotes growth of human thyroid tumor cells by different molecular mechanisms.* J Clin Endocrinol Metab, 2001. **86**(3): p. 1072-7.
77. Kumar, A., C.M. Klinge, and R.E. Goldstein, *Estradiol-induced proliferation of papillary and follicular thyroid cancer cells is mediated by estrogen receptors alpha and beta.* Int J Oncol, 2010. **36**(5): p. 1067-80.
78. Rajoria, S., et al., *Metastatic phenotype is regulated by estrogen in thyroid cells.* Thyroid, 2010. **20**(1): p. 33-41.
79. Pyo, J.S., J.H. Sohn, and G. Kang, *Detection of Tumor Multifocality Is Important for Prediction of Tumor Recurrence in Papillary Thyroid Microcarcinoma: A Retrospective Study and Meta-Analysis.* J Pathol Transl Med, 2016. **50**(4): p. 278-86.
80. Tam, A.A., et al., *Association of multifocality, tumor number, and total tumor diameter with clinicopathological features in papillary thyroid cancer.* Endocrine, 2016.
81. Qu, N., et al., *Risk Factors for Central Compartment Lymph Node Metastasis in Papillary Thyroid Microcarcinoma: A Meta-Analysis.* World J Surg, 2015. **39**(10): p. 2459-70.
82. Kaliszewski, K., et al., *Incidental and non-incidental thyroid microcarcinoma.* Oncol Lett, 2016. **12**(1): p. 734-740.
83. Pyo, J.S., et al., *The prognostic relevance of psammoma bodies and ultrasonographic intratumoral calcifications in papillary thyroid carcinoma.* World J Surg, 2013. **37**(10): p. 2330-5.
84. Lu, Z.Z., et al., *Outcome of papillary thyroid microcarcinoma: Study of 1,990 cases.* Mol Clin Oncol, 2015. **3**(3): p. 672-676.
85. de Biase, D., et al., *TERT Promoter Mutations in Papillary Thyroid Microcarcinomas.* Thyroid, 2015. **25**(9): p. 1013-9.
86. Melo, M., et al., *TERT promoter mutations are a major indicator of poor outcome in differentiated thyroid carcinomas.* J Clin Endocrinol Metab, 2014. **99**(5): p. E754-65.
87. Li, C., et al., *BRAF V600E mutation and its association with clinicopathological features of papillary thyroid cancer: a meta-analysis.* J Clin Endocrinol Metab, 2012. **97**(12): p. 4559-70.
88. Tallini, G., et al., *BRAF V600E and risk stratification of thyroid microcarcinoma: a multicenter pathological and clinical study.* Mod Pathol, 2015. **28**(10): p. 1343-59.
89. Virk, R.K., et al., *BRAFV600E mutation in papillary thyroid microcarcinoma: a genotype-phenotype correlation.* Mod Pathol, 2013. **26**(1): p. 62-70.
90. Lupi, C., et al., *Association of BRAF V600E mutation with poor clinicopathological outcomes in 500 consecutive cases of papillary thyroid carcinoma.* J Clin Endocrinol Metab, 2007. **92**(11): p. 4085-90.
91. Rodolico, V., et al., *BRAF V600E mutation and p27 kip1 expression in papillary carcinomas of the thyroid <or=1 cm and their paired lymph node metastases.* Cancer, 2007. **110**(6): p. 1218-26.
92. Frasca, F., et al., *BRAF(V600E) mutation and the biology of papillary thyroid cancer.* Endocr Relat Cancer, 2008. **15**(1): p. 191-205.
93. Kwak, J.Y., et al., *Association of BRAFV600E mutation with poor clinical prognostic factors and US features in Korean patients with papillary thyroid microcarcinoma.* Radiology, 2009. **253**(3): p. 854-60.

94. Lee, X., et al., *Analysis of differential BRAF(V600E) mutational status in high aggressive papillary thyroid microcarcinoma.* Ann Surg Oncol, 2009. **16**(2): p. 240-5.
95. Basolo, F., et al., *Correlation between the BRAF V600E mutation and tumor invasiveness in papillary thyroid carcinomas smaller than 20 millimeters: analysis of 1060 cases.* J Clin Endocrinol Metab, 2010. **95**(9): p. 4197-205.
96. Lin, K.L., et al., *The BRAF mutation is predictive of aggressive clinicopathological characteristics in papillary thyroid microcarcinoma.* Ann Surg Oncol, 2010. **17**(12): p. 3294-300.
97. Kurtulmus, N., et al., *BRAF(V600E) mutation in Turkish patients with papillary thyroid cancer: strong correlation with indicators of tumor aggressiveness.* Endocrine, 2012. **42**(2): p. 404-10.
98. Zhou, Y.L., et al., *Preoperative BRAF mutation is predictive of occult contralateral carcinoma in patients with unilateral papillary thyroid microcarcinoma.* Asian Pac J Cancer Prev, 2012. **13**(4): p. 1267-72.
99. Lim, J.Y., et al., *Clinicopathologic implications of the BRAF(V600E) mutation in papillary thyroid cancer: a subgroup analysis of 3130 cases in a single center.* Thyroid, 2013. **23**(11): p. 1423-30.
100. Rossi, E.D., et al., *BRAF (V600E) mutation analysis on liquid-based cytology-processed aspiration biopsies predicts bilaterality and lymph node involvement in papillary thyroid microcarcinoma.* Cancer Cytopathol, 2013. **121**(6): p. 291-7.
101. Zheng, X., et al., *Papillary microcarcinoma of the thyroid: clinical characteristics and BRAF(V600E) mutational status of 977 cases.* Ann Surg Oncol, 2013. **20**(7): p. 2266-73.
102. Yang, Y., et al., *Prediction of central compartment lymph node metastasis in papillary thyroid microcarcinoma.* Clin Endocrinol (Oxf), 2014. **81**(2): p. 282-8.
103. Bastos, A.U., et al., *BRAF V600E and decreased NIS and TPO expression are associated with aggressiveness of a subgroup of papillary thyroid microcarcinoma.* Eur J Endocrinol, 2015. **173**(4): p. 525-40.
104. Niemeier, L.A., et al., *A combined molecular-pathologic score improves risk stratification of thyroid papillary microcarcinoma.* Cancer, 2012. **118**(8): p. 2069-77.
105. Adeniran, A.J., et al., *Correlation between genetic alterations and microscopic features, clinical manifestations, and prognostic characteristics of thyroid papillary carcinomas.* Am J Surg Pathol, 2006. **30**(2): p. 216-22.
106. Piana, S., et al., *Papillary thyroid microcarcinoma with fatal outcome: evidence of tumor progression in lymph node metastases: report of 3 cases, with morphological and molecular analysis.* Hum Pathol, 2013. **44**(4): p. 556-65.