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Utilization of a repository of thyroid tumours: clinico-pathological and molecular characterization

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Utilization of a repository of thyroid tumours: clinico-pathological and molecular characterization

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

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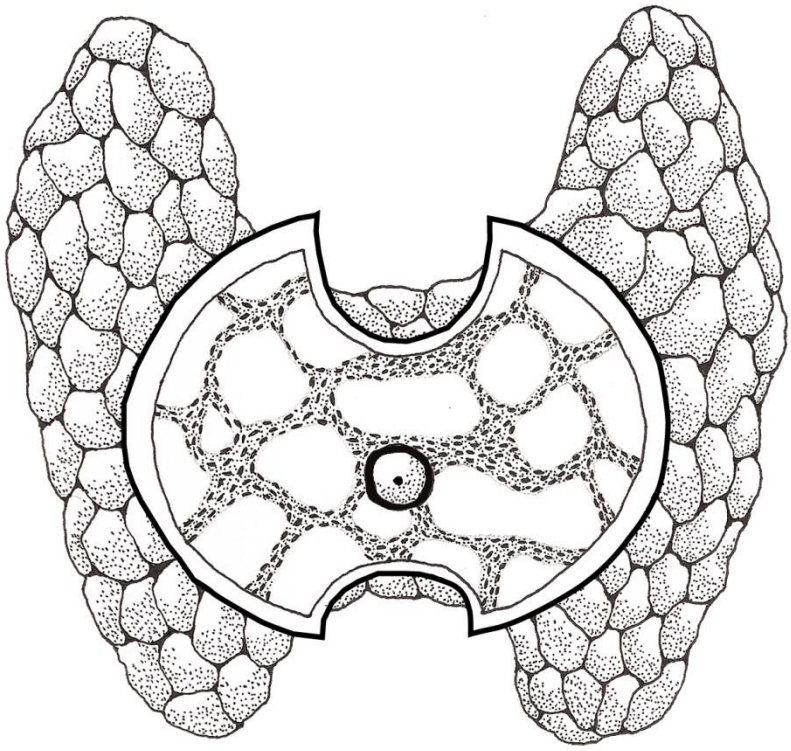

“Não me venham com conclusões! A única conclusão é morrer.”

Álvaro de Campos

Parte dos resultados originais desta tese de mestrado são parte integrante dos seguintes trabalhos já publicados:

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Abbreviations

CCDC6 – coiled-coil domain containing 6

cPTC – classic papillary thyroid carcinoma

DAPK – death-associated protein kinase

Diffuse sclerosing PTC – diffuse sclerosing variant of papillary thyroid carcinoma

DNA – deoxyribonucleic acid

DTC – differentiated thyroid carcinoma

FTA – follicular thyroid adenoma

FTC – follicular thyroid carcinoma

FVPTC – follicular variant of papillary thyroid carcinoma

GDNF – glial-derived neurotrophic factor

HRAS – Harvey rat sarcoma viral oncogene homolog

hTERT – human telomerase reverse transcriptase gene

KRAS - Kirsten rat sarcoma viral oncogene homolog

MAPK – mitogen-activated protein kinase pathway

MTC – medullary thyroid carcinoma

NCOA4 – nuclear receptor coactivator 4

NIS – sodium iodide symporter

NF-kB – nuclear transcription factor kB

NRAS - neuroblastoma rat sarcoma viral oncogene homolog

Oncocytic FTA – oncocytic variant of follicular thyroid adenoma

Oncocytic FTC – oncocytic variant of follicular thyroid carcinoma

Oncocytic PTC – oncocytic variant of papillary thyroid carcinoma

PAX8 – paired box 8

PCR – polymerase chain reaction

PDTC – poorly differentiated thyroid carcinoma

PI3/AKT – phosphatidylinositol 3-kinase/AKT pathway

PPARG – peroxisome proliferator-activated receptor gamma

PPFP – PAX8-PPARɣ fusion protein

PRKAR1A – regulatory subunit type I α of protein kinase A

PTC – papillary thyroid carcinoma

qRT-PCR – real time polymerase chain reaction

RAF – rapidly accelerated fibrossarcoma

RARβ2 – retinoic acid receptor β2

RAS – rat sarcoma

RET – rearranged during transfection

RNA – ribonucleic acid

RT-PCR – reverse transcriptase polymerase chain reaction

SCNs – solid cell nests

SVPTC – solid variant of papillary thyroid carcinoma

Tall cell PTC – tall cell variant of papillary thyroid carcinoma

T3 – triodothyronine

T4 – thyroxine

TIMP3 – tissue inhibitor of matrix metalloproteinase-3

TFC – thyroid follicular cells

UTC – undifferentiated thyroid carcinoma

Resumo

O cancro da tiróide é a neoplasia endócrina mais frequente, representando cerca de 1% de todos os cancros, sendo mais comum em mulheres. Os tumores da tiróide compreendem um largo espectro de lesões benignas e malignas, mostrando diferenças morfológicas e de prognóstico. Nos útlimos anos foi alcançado um avanço significativo no conhecimento dos mecanismos moleculares da carcinogénese e progressão do cancro da tiróide, onde mutações recorrentes em cancro da tiróide foram associadas com fenótipos tumorais específicos e implicados na etiologia da doença. A ativação da via *mitogen-activated protein kinase* (MAPK) e da *phosphatidylinositol 3-kinase* (PI3)/AKT, frequentemente em estreita ligação e cooperação, constituem os principais mecanismos para o desenvolvimento e progressão da maioria dos tumores da tiróide.

O objectivo deste trabalho centrou-se na organização de um repositório de tumores da tiróide e construção de uma base de dados, proporcionando um acesso facilitado a amostras de DNA, RNA e proteínas de alta qualidade, com os principais dados clínico-patólogicos e análise molecular de cada caso, para utilização em estudos futuros no grupo de investigação.

Os protocolos standard estabelecidos para a extracção de ácidos nucleicos revelaram uma taxa de sucesso acima de 97.0% e a base de dados construída incluiu 54 amostras de bócios nodulares, 24 amostras de adenomas foliculares, 117 amostras de tumores malignos e 30 amostras de tiróides normais.

Considerando todas as amostras de tumores analisadas, 26 tumores (21.1%) eram positivos para mutações no *BRAF,* 20 tumores (14.9%) para mutações no *NRAS,* 4 tumores (2.9%) para mutações na região promotora do *TERT*, 12 tumores (9.1%) eram positivos para os rearranjos *RET/PTC1,* 2 tumores (1.5%) para os rearranjos do *RET/PTC3,* e 2 tumores (1.5%) para rearranjos *PAX8-PPARɣ*.

Várias diferenças estatisticamente significativas foram encontradas na análise das caracteristicas clínico-patológicas e moleculares, quando os tumores foram agrupados por diagnóstico e comparados.

A organização do repositório de material biológico de tumores da tiróide e a construção da base de dados foram objectivos cumpridos, com o estabelecimento de protocolos standard. As perspectivas futuras centram-se na organização dos restantes casos no repositório, a caracterização clinico-patológica completa, com revisão histológica, da totalidade das lesões do repositório e com a análise rápida das alterações genéticas mais conhecidas em tumores da tiróide.

Abstract

Thyroid cancer is the most frequent endocrine neoplasia, accounting for about 1% of all human cancers being more frequent in women than in men. Thyroid tumours comprise a spectrum of benign and malignant lesions showing very diverse morphologic and prognostic differences. In recent years a significant knowledge in molecular mechanisms of thyroid carcinogenesis and progression has been achieved, where recurrent mutations in thyroid carcinomas are predominantly associated with specific tumour phenotypes and are implicated in the disease aetiology. While several molecular alterations are implied in thyroid carcinogenesis, the activation of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/AKT pathways, commonly in close connection and cooperation, constitute the major mechanisms for the development and progression of most thyroid tumours.

The challenge for this work was the organization of a repository of thyroid tumours and construction of a new database, providing an easier availability to DNA, RNA, and protein components with high quality, with the main clinico-pathological and molecular data available for each case, to be used for furthers studies in the research group.

The established standard protocols for the extraction of the nucleic acids revealed a success rate above 97.0% and the database included 54 samples of nodular goiter, 24 samples of follicular thyroid adenoma, 117 samples of malignant tumours and 30 samples of normal thyroid.

Considering all tumour samples analysed, 26 (21.1%) were positive for the *BRAF* mutations, 20 tumours (14.9%) were positive for *NRAS* mutations, 4 tumours (2.9%) were positive for *TERT* promoter mutations, 12 tumours (9.1%) were positive for *RET/PTC1* rearrangement, 2 tumours (1.5%) were positive for *RET/PTC3* rearrangement and 2 tumours (1.5%) were positive for *PAX8-PPARɣ* rearrangement.

Several statistically significant differences were found in the analysis of clinico-pathological and molecular features, when the tumours were grouped by diagnosis and compared.

The organization of the repository of biological material of thyroid tumours and database was an achieved purpose with the establishment of successful standard protocols. The future perspectives include the organization of the remaining cases from the repository, complete clinico-pathological characterization, with histological revision, and the screening of the most common genetic alterations known in thyroid tumours.

Introduction

Thyroid cancer is the most frequent endocrine neoplasia, accounting for about 1% of all human cancers (1) being more frequent in women than in men (2). With the improvement of the detection methods the incidence of thyroid cancer has been steadily increasing, about 6.6% *per* year, worldwide over the last few decades (3).

In Portugal, thyroid cancer is the third most frequent cancer in women according to the Oncologic Regional Registry of the North database [*Registo Oncológico Regional do Norte* (RORENO)] (4). Thyroid cancer may occur at any age, being the peak incidence 45 to 49 years in women and 65 to 69 years in men (5).

Even though the death rate of thyroid cancer is relatively low, the social and economic impact of this disease, is a major concern since the rate of disease recurrence or persistence is high (6).

In recent years, research has been directed to personalized medicine, where the availability of banks of biological material with large series of biospecimens are important tools for the identification/validation of novel parameters with clinical impact (7). A bank of biological material collects a high variety of biological samples, including tumour/non tumour tissues, cells, blood, plasma and other body fluids, DNA and RNA (8). The availability of high quality human samples is fundamental in the modern molecular medicine for the validation of novel signalling pathways and to personalized therapeutic regimens for treatment (9).

In oncological investigation, sample collections with high quality DNA, RNA and proteins, together with relevant clinical, molecular and pathological data, are a gold standard to ensure the accuracy and validation of biomarkers for diagnostic and prognostic purposes, or for the identification of genes for targeting therapy.

Thyroid tumours

The thyroid is mainly composed by differentiated epithelial cells known as thyroid follicular cells (TFC) that are responsible for the production and export of thyroid hormones, such as triiodothyronine (T3) and thyroxine (T4), essential for growth, development and survival. More than 95% of thyroid cancer cases are originated from TFC, and can be classified in several histological types and subtypes with different characteristics and prognoses (10).

Thyroid tumours comprise a spectrum of benign and malignant lesions showing very diverse morphologic and prognosis differences. In one hand of the spectrum we have the follicular thyroid adenoma, a benign encapsulated lesion, and in the other end we have a highly invasive tumour the undifferentiated thyroid carcinoma.

Epithelial thyroid malignancies can be divided in differentiated thyroid carcinomas (DTC), which include papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC), poorly differentiated thyroid carcinomas (PDTC) and undifferentiated thyroid carcinomas (UTC) (10).

Medullary thyroid carcinoma (MTC), originated from the parafollicular C-cells, accounts for a small proportion (5%) of all thyroid malignancies (10).

We will now describe the main histological variants of thyroid tumours emphasising those that will be addressed in our analysis.

Follicular thyroid adenoma

Follicular thyroid adenoma (FTA) is a benign lesion of the thyroid gland, defined as an encapsulated tumour showing evidence of follicular cell differentiation. Typically is enclosed in a fibrous capsule of variable thickness and by definition, capsular or vascular invasion are absent (11). The architectural patterns and cytological features are different from those of the surrounding thyroid tissue.

The differential diagnosis between FTA and FTC is based on the presence of vascular and/or capsular invasion, which determines the importance of tumour sampling (11).

Several histologic variants of FTA have been described besides the conventional FTA.

Oncocytic thyroid adenoma

The oncocytic FTA is composed by cells with abundant granular eosinophilic cytoplasm and large open nuclei, with a variety of architecture patterns ranging from well-formed follicles to solid and/or trabecular growth (12). The nuclear criteria excludes the oncocytic variant of PTC and the lack of capsular or vascular invasion excludes the oncocytic variant of follicular thyroid carcinoma (oncocytic FTC) (11).

They are often associated with Hashimoto thyroiditis, and can occur particularly in young female patients and are associated with a risk of progression to carcinoma (13).

Fetal thyroid adenoma

The fetal thyroid adenoma is characterized by a microfollicular/trabecular structure in an oedematous stroma, particularly in the centre of the tumours (14).

Papillary thyroid carcinoma

PTC account for more than 85% of DTCs, and is by definition a malignant epithelial tumour showing evidence of follicular cell differentiation, being characterized by distinctive nuclear features (15), such as large and clear nuclei, with cytoplasmatic inclusions and grooves (16).

When the PTC, besides the characteristic nuclear features, are composed totally or in part by papillae it is classified as a classic variant of PTC (cPTC), that is the more common histotype. There are several other histological subtypes of PTC described, besides the cPTC. Always presenting the characteristic nuclei, the variants of PTC differ in the morphologic organization and background (15).

Follicular variant of papillary thyroid carcinoma

The follicular variant of PTC (FVPTC) often resembles encapsulated follicular neoplasm. They are composed of small follicles with virtually no papillary structures (less than 5%), however the nuclear changes typical of PTC allows this variant to be distinguished from the other follicular patterned lesions (15). The FVPTC may appear as encapsulated or infiltrative/diffuse tumours (17). The first form rarely metastasize to lymph node, only in around 5% of cases, whereas the infiltrative variants often harbour lymph node metastasis, which is reported in around 65% of cases. The prognosis of these tumours is similar to the cPTC (17).

Diffuse sclerosing variant of papillary thyroid carcinoma

The diffuse sclerosing variant of PTC (diffuse sclerosing PTC) is a rare variant of PTC characterized by diffuse involvement of one or both thyroid lobes, usually without forming a dominant mass (15). In the presence of dominant nodules, the neoplastic cells appear similar to those of cPTC and occasionally show a predominant follicular pattern. They tend to occur in younger patients, may harbour prominent regional node metastases and lung metastases can also be present at presentation (15).

Tall cell variant of papillary thyroid carcinoma

The tall cell variant of PTC (tall cell PTC) is an uncommon variant of PTC. It is predominantly composed of cells whose heights are at least three times their widths. This variant is composed of a combination of papillary, trabecular or cord-like patterns (15).

These tumours occur in older patients, often males, and tend to show a more aggressive clinical behaviour than cPTC (18).

Solid variant of papillary thyroid carcinoma

The solid variant of PTC (SVPTC) is characterized by the presence of solid sheets of tumours cells with typical nuclear features of PTC, and it is only is considered when more than 50% of the neoplastic cell present a solid growth pattern (19). These tumours are more frequent in children, including those who have been exposed to radiation (15).

Oncocytic variant of papillary thyroid carcinoma

The oncocytic variant of PTC (oncocytic PTC) is characterized by the presence of mitochondria-rich follicular cells presenting eosinophilic and granular cytoplasm – oncocytic cells – that may have a papillary or follicular architecture (20, 21). The diagnosis of the oncocytic PTC is based on the nuclear features of these lesions which are identical to those seen in cPTC (15). These tumours can present well circumscribed, however some degree of infiltration of the surrounding capsule can be found, and they can be widely invasive (15).

Follicular thyroid carcinoma

FTCs account for about 5-10% of the DTC (22). FTCs are identified by their follicular organization and by the lack of PTC nuclear features. The differences between FTCs and FVPTCs are not always clear. The diagnosis of FTC also depends on evidence of vascular or capsular invasion in the tissue sample, and therefore occasionally a minimally invasive FTC will mistakenly be classified as a FTA. Molecular markers that can distinguish minimally invasive FTCs from FTAs have proved elusive but are continuously being sought (23).

Oncocytic variant of follicular thyroid carcinoma

Oncocytic FTC is a subtype of FTC, that accounts for 3-4% of thyroid cancer and is characterized by large, mitochondria-rich oncocytic cells that represent more than 75% of the cells, and dense nuclei and nucleoli (24, 25).

This variant also has a high propensity for metastasis and a poor prognosis (26), harbouring nodal metastases in approximately 30% of cases or distant metastases involving lung and bone (27).

Poorly differentiated thyroid carcinoma

PDTC represent less than 5% of thyroid carcinomas (22), and by definition are follicular-cell neoplasms that show limited evidence of follicular cell differentiation and occupy both morphologically and behaviourally an intermediate position between DTCs and UTCs (28). For the PDTC diagnosis, several criteria have to be fulfilled, the tumour has to include presence of a solid/trabecular/insular pattern of growth; absence of the conventional nuclear features of papillary carcinoma; and presence of at least one of the following features: convoluted nuclei; mitotic activity >or=3 x 10 HPF; or tumour necrosis. An algorithmic approach was devised for practical use in the diagnosis of this tumour (29).

Undifferentiated thyroid carcinoma

UTC is the deadliest subtype of thyroid cancer. It is rare, accounting for at most 2% of cases and causes up to 14-50% of thyroid cancer deaths (30). UTCs, also called anaplastic thyroid carcinoma, are highly malignant tumours composed of undifferentiated

cells that exhibit immunohistochemical or ultra-structural features indicative of epithelial differentiation. They are usually large, locally invasive, and consist of admixtures of spindle, epithelioid and pleomorphic giant cells (31). The fact that UTCs are massively infiltrated with macrophages may contribute in part to their heterogeneous appearance (32, 33).

Medullary thyroid carcinoma

MTC is a malignant tumour that harbours C-cell differentiation. Histologically the MTC presents sheets, nests or trabeculae of polygonal, round or spindle cells, separated by varying amounts of fibrovascular stroma, giving rise to more or less lobular or trabecular arrangement (34-36).

This malignant tumour tends to metastasize early, particularly to cervical lymph nodes, and haematogenous MTC tends to metastasize to liver, lungs and bone, and occasionally to the brain, soft tissues around the neck and to the bone marrow (37).

Genetic alterations in thyroid tumours

In recent years a significant knowledge in molecular mechanisms of thyroid carcinogenesis and progression has been achieved, where recurrent mutations in thyroid carcinomas are predominantly associated with specific tumour phenotypes and are implicated in the disease aetiology. While several molecular alterations are implied in thyroid carcinogenesis, the activation of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/AKT pathways, commonly in close connection and cooperation, constitute the major mechanisms for the development and progression of most thyroid tumours (38).

The MAPK pathway (Figure 1) has a fundamental role in the regulation of cell growth, proliferation, apoptosis and metabolic activities through regulation of the expression of several genes, while the PI3K-AKT (Figure 2) pathway also plays a similar role in cell regulation (39, 40). Nevertheless, activation of these pathways is attributed to different genetic or epigenetic alteration of oncogenes and tumours suppressor genes. In thyroid cancer, these pathways are driven by genetic alterations that are found in 65-70% of the TFC derived tumours, that include *BRAF*, *RAS* and *TERT* mutations, *RET/PTC* and *PAX8-PPARγ* rearrangements, and in some cases by the recently discovered *ALK* mutations (41-44). Mutations in tumour suppressor gene *p53* are the major event in the UTC (45).

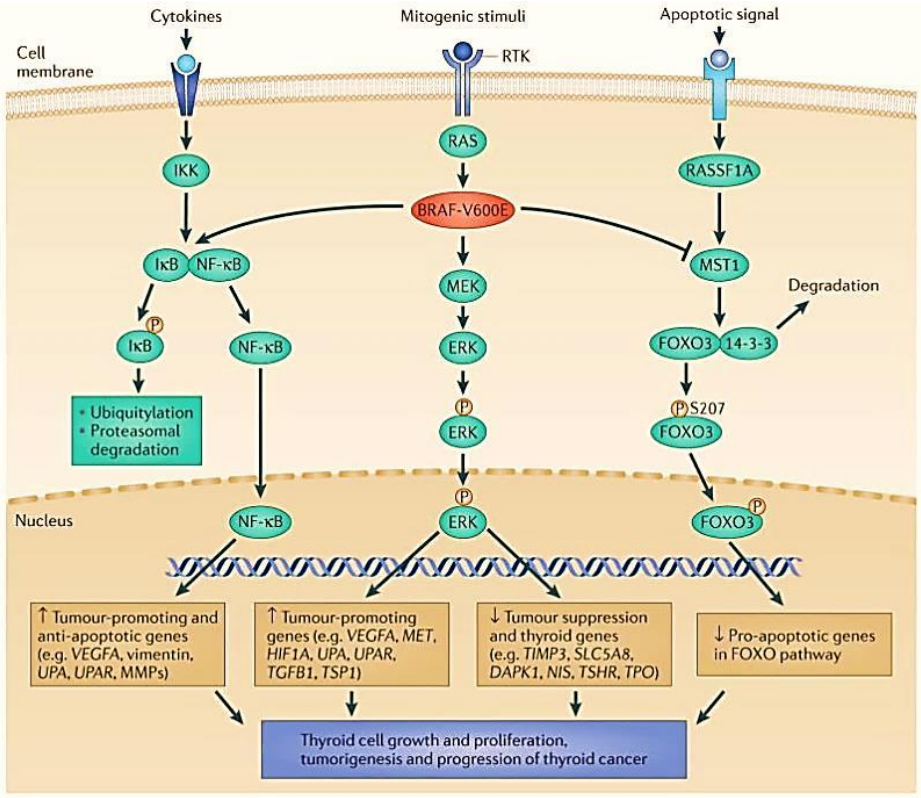

Figure 1 - *The MAPK and related pathways in thyroid cancer. Adapted from: Xing, M. 2005. Molecular pathogenesis and mechanisms of thyroid cancer.* Nat Rev Cancer. *13(3):184-99.*

BRAF mutations

BRAF, a proto-oncogene located at 7q24, is a member of RAF kinase family proteins, which are intracellular effectors of the MAPK pathway signalling cascade. Their activation is triggered by RAS binding and protein recruitment to the cell membrane which will stimulate its serine/threonine kinase activity, and result in phosphorylation and activation of MEK, which in turn activates ERK and consequent effectors of the MAPK cascade (46, 47).

All three functional human RAF proteins – A-RAF, B-RAF and C-RAF- share several highly conserved regions in the N-terminal regulatory domain and C-terminal kinase domain. Of all RAF proteins, BRAF has the highest basal kinase activity and is more active in phosphorylating MEK, since it has higher affinity for MEK 1 and 2 and is more efficient in phosphorylating MEKs upon RAS activation than the other two family members (46).

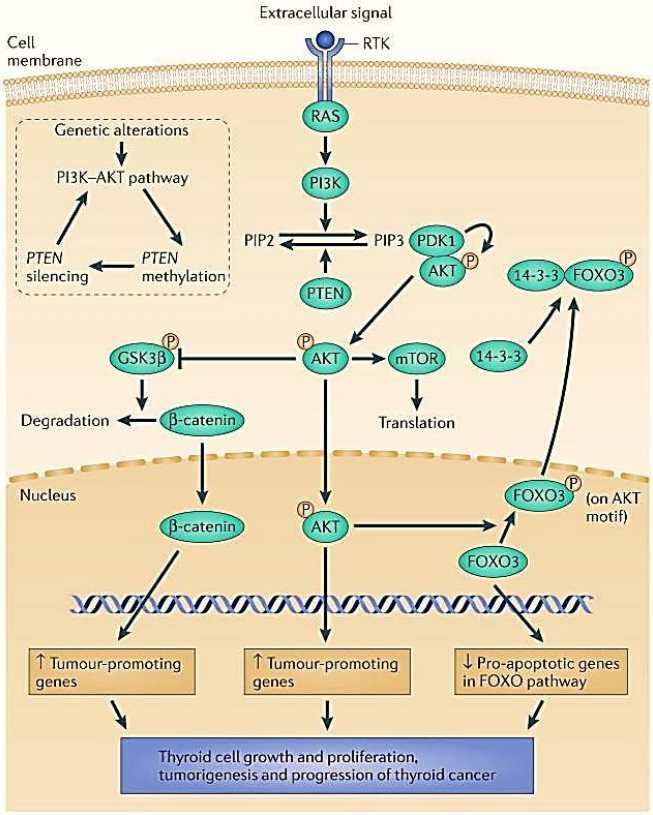

Figure 2 - *The PI3K-AKT and related pathways in thyroid cancer. Adapted from: Xing, M. 2005. Molecular pathogenesis and mechanisms of thyroid cancer. Nat Rev Cancer. 13(3):184-99.*

BRAF gene alterations were associated with human carcinogenesis after the description of a high frequency of *BRAF* activating mutations in melanomas, colorectal and ovarian carcinomas (48). All mutations described occurred within the kinase domain of the protein, involving either the activation loop or the ATP binding site (48), disrupting the interaction between both lobes of the protein and destabilizing the inactive conformation of the kinase, and resulting in BRAF constitutive activation. Most, but not all, know oncogenic *BRAF* genetic alterations allow the formation of new interactions that fold the kinase into a catalytically competent structure (49).

A wide variety of *BRAF* mutations are found in a wide range of cancers, but more than 95% of the cases are of a single variety denoted *BRAF* (p.V600E). This mutation was characterized for the first time in thyroid carcinomas in 2003 (41, 50, 51). It is a missense thymine to adenine transversion at the 1799 nucleotide position in exon 15 that leads to a valine to glutamate substitution at the 600 amino acid position, in the kinase domain (52, 53). The mutation results in a constitutive activation of the BRAF, no longer dependent on RAS, and hence causes constitutive MEK/ERK activation (52, 53).

In 1% to 2% of PTC, other *BRAF* mutations can be found such as *BRAF* (p.K601E) point mutation, small in-frame insertions or deletions surrounding codon 600 (54) and a

chromosomal rearrangement resulting in the fusion gene *AKAP9-BRAF*, that was found in PTCs associated with radiation exposure (55).

The *BRAF* (p.K601E) mutation consists in a missense adenine to guanine transversion at the 1801 nucleotide position that leads to a lysine to glutamate substitution at the 601 amino acid position (48), and has a similar effect than the *BRAF* (p.V600E) mutation.

BRAF mutations are now thought to be the most common mutation in PTC, with an incidence of 45%, with the highest frequencies in tall cell PTC (80%), cPTC (60%) and less frequent in FVPTC (10%) (56). In PTC the most common mutation is the *BRAF* (p.V600E), however in the FVPTC the *BRAF* (p.K601E) is frequently found (57).

BRAF mutations are more common in PTC of adults than in PTC from children (58), and are rare in radiation-induced PTCs (59).

An initiator role for *BRAF* mutation in PTC has been advanced by the demonstration that thyroid-targeted *BRAF* (p.V600E) transgenic mice develop thyroid tumours with PTC features (60), and by the frequent detection of *BRAF* mutations in papillary thyroid microcarcinomas (20-52%) (57). *BRAF* (p.V600E) induces genomic instability (61), facilitating the acquisition of secondary genetic events that promote tumour progression (62). Early or late expression of *BRAF* (p.V600E) in transgenic mice rapidly produces enlarged and abnormal thyroids followed by invasive PTC within a year, confirming *BRAF* mutations as a causative event in PTCs (60, 63).

BRAF is frequently associated with aberrant methylation of several tumour suppressor genes, such as the *tissue inhibitor of matrix metalloproteinase-3* (*TIMP3*), the *death-associated protein kinase* (*DAPK*), *SLC5A8*, and *retinoic acid receptor β2* (*RAR β2*) (64). *In vitro* studies have demonstrated that *BRAF* (p.V600E) confers enhanced invasiveness to thyroid cells, through increased expression of matrix metalloproteinase 3, 9 and 13, VEGF and by activation of the nuclear transcription factor kB (*NF-kB*) – coupled signalling, which promotes apoptosis resistance, cell proliferation, angiogenesis, invasion and metastasis (64). The enhanced invasiveness may explain why thyroid *BRAF* (p.V600E) transgenic mice develop more invasive PTCs (60) that frequently evolve to PDTCs and supports the role of *BRAF* in tumour progression.

Clinical studies have demonstrated *BRAF* association with extrathyroidal extension, lymph node metastasis, advanced tumour stages, older age at diagnosis, disease recurrence, and even patient mortality when associated with other features of bad prognosis (65). These data have been interpreted as indicative of poorer prognosis in *BRAF*-mutated thyroid carcinoma (66).

There is also recent evidence showing an association between the presence of *BRAF* mutations and PTC recurrence, mortality and resistance to radioiodine therapy (67-

69), that is probably related to the association of *BRAF* mutations with silencing of iodine-handling genes (70), and to the demonstration that *BRAF*-mutated PTC may evolve to PDTC and UTC (71). The more aggressive behaviour of *BRAF* (p.V600E) positive tumours may be due to the propensity of these tumours to dedifferentiate, and to the fact that this mutation leads to the alteration of the function of *sodium iodide symporter* (*NIS*) and other genes metabolizing iodide, thus decreasing the ability of tumour cells to trap radioiodine and predisposing to treatment failure and recurrent disease (44, 69-71).

However, recent studies have challenged this concept, by demonstrating that, when confounding factors are accounted for by means of a multivariate statistical analysis, *BRAF per se* is not correlated with bad prognostic features. As a result, a recent review has concluded that there is still not enough evidence to warrant a more aggressive approach to thyroid carcinomas harbouring *BRAF* mutations, given the high general incidence of *BRAF* mutations would put as many as 30% of all PTC patients at risk of overtreatment (64).

More importantly, *BRAF* (p.V600E) has been found to be an independent predictor of treatment failure and tumour recurrence, even in patients with low-stage disease (44, 72, 73), and as an risk factor for tumour-related death (74).

BRAF mutations have also been described in 10 to 44% of UTC (69, 75, 76); in such *BRAF*-mutated UTC, the mutations are frequently also detected in adjacent foci of PTC that are thought to represent the origin of UTC. This supports the idea that *BRAF* mutations can be implicated in the progression of DTC to PDTC or UTC (44, 77).

In accordance with a stepwise progression model, *BRAF* mutations are more frequent in PDTC arising from PTC, than in PDTC associated with FTC (75, 76). This was clear when Soares *et al.* evaluated a group of PDTC exclusively composed of insular and insular-like tumours, thus excluding PDTC with foci displaying PTC nuclei (76). No *BRAF* mutations were detected in this group, supporting the assumption that pure and insular-like PDTC are more closely related to FTC than to PTC (76). *BRAF* mutations were nevertheless described in PDTC with PTC-like nuclei, as well as in PDTC coexisting with foci of PTC (69, 75).

NRAS mutations

The RAS proteins are plasma membrane GTPases that regulate key cellular processes involved in growth, differentiation, survival, adhesion and migration (47, 78, 79). *RAS* genes encode highly related G proteins that are located at the inner surface of the cell membrane and propagate signals arising from the cell membrane receptor tyrosine kinase and G-protein-coupled receptors along the MAPK, PI3K/AKT, and other signalling pathways. RAS exists in a guanosine triphosphate (GTP)-bound active state or a guanosine diphosphate (GDP)-bound inactive state, and RAS activation is induced by external signals

such as from cell surface receptor tyrosine kinases. Constitutively activating *RAS* mutations are one of the most common mutations in cancers (80).

The three members of the *RAS* family, *HRAS*, located on chromosome 11p11, *KRAS*, located on chromosome 12p12 and *NRAS*, located on chromosome 1p12, have been shown to be mutated in thyroid cancer, where they become constitutively activated either by mutations that enhance their GTP-binding affinity (codons 12/13) or that decrease their intrinsic GTPase activity (codon 61) (78, 79).

Overall, the most frequent mutations in thyroid cancer lie in codon 61 of *NRAS* and, less commonly, codon 61 of *HRAS (79)* . In the Switch II region of the G domain, in codon 61, several mutations can be found, for example the *NRAS* (p.Q61R) mutation that is a missense adenine to guanine transversion at the 182 nucleotide position that leads to glutamine to an arginine substitution. The *NRAS* (p.Q61K) mutation is a missense cytosine to adenine transversion at the 181 nucleotide position that leads to a glutamine to a lysine substitution at the 61 amino acid position. Both mutations will lead to a constitutive activation of the NRAS, even in the absence of growth factor signalling (78, 79, 81).

In thyroid tumours, *RAS* mutations are more common in iodine-deficient areas (81) and rare in radiation-induced thyroid cancers of Chernobyl (82). *RAS* mutations are present in 20-40% of FTA, 40-50% of FTCs, and 10-20% of PTCs (the majority in the FVPTC) with the following relative frequency: *NRAS*> *HRAS*> *KRAS* (83).

RAS mutations were thought to be early events in thyroid carcinogenesis, due to their presence in both FTAs and FTCs; however this may result in part from the high degree of inter observer variability concerning the distinction of FTA from FTC (84, 85).

In addition, recent studies have convincingly shown lower rates of *RAS* mutations in DTCs and higher in PDTCs and UTCs, suggesting a role of *RAS* in tumour progression, rather than initiation (86, 87) . This is supported by *in vitro* observations of increased genomic instability induced by *RAS* – genomic instability is believed to be a key step in tumour progression by allowing tumour cells to accumulate mutations that promote increased survival and invasiveness (88). Moreover, an animal model of thyroid-targeted *RAS* mutation resulted in follicular thyroid tumours that progressed to PDTCs (89). Nevertheless, *RAS* mutations are also prevalent in FTA, and are seen in FVPTC with good clinical behaviour and with few metastases (90-92).

RAS mutations have been detected in 18-27% of PDTCs and up to 60% of UTCs; however the prognostic value of *RAS* mutations in thyroid cancer is not well established. In the more aggressive tumours, such as PDTC and UTC, some series showed that *RAS* mutations were associated with aggressive tumour phenotypes and poor prognosis (86, 87), whereas in others series such associations were not observed (69). It has been advanced that PDTC and UTC tumours with mutated *NRAS* are significantly associated

with the appearance of haematogenous (particularly bone) metastases, suggesting a role of *RAS* gene activation in the metastatic capability of these tumours (87, 93, 94).

***RET/PTC* rearrangements**

The *RET* (*rearranged during transfection*) proto-oncogene is located on chromosome 10q11.2 and encodes a single-pass transmembrane tyrosine kinase that has an extracellular ligand-binding domain with four cadherin-like repeats and a cysteine-rich region, a hydrophobic transmembrane domain, and a cytoplasmic tyrosine kinase domain (95). This peculiar organization defines RET as distant member of cadherin superfamily and raises the possibility that *RET* may have arisen by a recombination of an ancestral cadherin with a tyrosine kinase gene (95).

The structure of RET implicated it as a transmembrane receptor; however it remained an orphan receptor until a knockout mouse model deficient in *glial-derived neurotrophic factor* (*GDNF*) was found to exhibit a phenotype identical to *RET* -/- mice (96, 97). It was then established that RET functions as the receptor for GDNFs, which activate RET when bound to GDNF-family receptor-α (GFRα) and that both are essential for the development of the sympathetic, parasympathetic, and enteric nervous system, the kidney and the testis (95).

In PTC, *RET*-related carcinogenesis occurs not by gain-of-function mutations, but through the action of fusion proteins generated by rearrangement of *RET* that fuse the tyrosine kinase domain of *RET* with the 5'-terminal region of unrelated genes. These have been collectively designated *RET/PTC* rearrangements. The first *RET/PTC* rearrangement was discovered in 1987 (98), and since then, at least 11 rearranged forms of *RET* have been described (99, 100), and others have recently been reported (101, 102). *RET/PTC1* (alias *CCDC6-RET*) results from a paracentric chromosomal inversion inv(10)(q11.2q21), leading to the fusion of *RET* and *CCDC6* (alias *H4)*; and *RET/PTC3* (alias *NCOA4-RET)* results from a paracentric inversion, inv(10)(q11.2;q11) that fuses *RET* and *NCOA4* (alias *ELE1*) (103, 104).

RET/PTC1 is the most common rearrangement type and comprises up to 60-70% of all positives PTC cases (105). *RET/PTC3* accounts for 20 to 30% and *RET/PTC2* and other novel rearrangements types for fewer than 5% of all detected rearrangements in PTC (106, 107).

RET/PTC rearrangements place *RET* under the transcriptional control of its fusion partner gene promoter, which allows the expression of RET in TFC, where it is normally not expressed. The rearrangement also deletes the signal sequence, the extracellular ligand-binding domain and the intracellular juxta membrane domains of the receptor, re-locating the RET/PTC protein to the cytosolic compartment, and preventing it from interacting with

many of its negative regulators. In addition, the presence of coiled-coil domains (protein-protein interaction domains able to mediate dimerization) in *RET* fusion partners result in ligand-independent dimerization and phosphorylation of RET downstream targets, with constitutive activation of the RAS/MAPK pathway and the PI3K/AKT pathways (105, 108-111). The altered function of *RET* fusion partners, which have so far not been extensively studied, may also account for some of the oncogenic properties (105). Altered function of *RET* fusion partners may also explain why the *RET/PTC* variants have different biological activities. Some examples are *RET/PTC2*, whose fusion partner, the *regulatory subunit type I α of protein kinase A* (*PRKAR1A*) is a tumour suppressor, similarly, *CCDC6* when overexpressed induces apoptosis, suggesting that abrogation of this function may facilitate PTC cell survival (112).

RET/PTC rearrangements have so far been identified only in thyroid lesions, in particular PTC cases, where their prevalence ranges from 13-46%, depending on the detection method used and the geographic region studied (56). It has been reported that *RET* rearrangements are more frequent in tumours with classic architecture, and rare in the FVPTC (113).

A high prevalence of *RET/PTC* mutations is found in tumours from patients who received external radiation and in post-Chernobyl PTCs (over 60%). Among those, *RET/PTC3* was more prevalent in short latency PTCs, whereas *RET/PTC1* was more frequently found in latter latency and sporadic PTCs (114). Corroborating these observations, *in vitro* studies have confirmed a causative role for X-ray irradiation in the formation of *RET/PTC* rearrangements (115). This might be favoured by the fact that the most common partners (*NCOA4* and *CCDC6*) lie in closer proximity in interphase thyroid cells than would be expected by their genomic localization (116) due to large-scale chromosome folding in the region of chromosome 10 spanning these loci (117).

RET/PTC1 tumours are associated with cPTC histology while *RET/PTC3* are associated with more aggressive SVPTC (103, 114, 118).

The role of *RET/PTC* rearrangements as early events in thyroid carcinogenesis is supported by the finding of *RET/PTC* rearrangements in a high percentage of papillary thyroid microcarcinomas (119, 120) as well in nodules with incomplete morphological evidence of PTC (121). In addition, introduction of *RET/PTC* retroviral constructs into thyroid epithelial cells leads to development of typical nuclear morphology that is diagnostic of PTC (122). Thyroid targeting of *RET/PTC1* (104, 123) or *RET/PTC3* (124) in transgenic mice leads to the development of tumours that resemble PTC. In non-malignant primary thyroid cells or cell lines, *RET/PTC* expression decreases thyroid specific gene expression, alters cell and colony morphology to resemble features of PTC, renders cell insensitive or

independent from TSH signalling, and especially in the cases of *RET/PTC3*, markedly increases cell proliferation (103, 125, 126).

It has been suggested that *RET*-associated PTCs are phenotypically indolent, rarely exhibit lymph node invasion or distant organ metastases (124) and have a very low probability of progression to PDTC and UTC (107). Although few correlations have been made between *RET/PTC* rearrangements and clinic-pathological features, there are some studies that observed increased lymph node metastases in these cases (107, 113, 127).

This lack of poor prognosis is in striking contrast, however, to the *RET*-related aggressive MTCs. *RET* mutations are frequently found in MTC, in 98% of hereditary MTCs a germline point mutation of *RET* is identified and in 30-70% of sporadic MTCs somatic mutations are found (128). *In vitro* studies show that both genetic alterations, the rearrangement and point mutations, activate similar downstream signalling pathways, and that the cytoplasmic location of *RET/PTC* actually increases its stability, by protecting it from receptor-mediated endocytosis and degradation. One possible explanation lies in the fact the transcript levels of *RET/PTC* were shown to be lower than the full-length *RET*, which is probably due to a relative weakness of the *RET/PTC1* and *RET/PTC3* in thyroid C-cells (129).

RET/PTC rearrangements have also been described in tumours with follicular pattern, particularly in oncocytic FTA and oncocytic FTC (12, 130, 131), with frequencies of 30% in oncocytic FTA and 38% for the oncocytic FTC, where all positive tumours displayed a solid growth pattern (132).

The frequency of *RET/PTC* in PDTC has been reported to be considerably lower than in PTC (13-17%) (69, 127) or absent (87, 107). Moreover the few *RET/PTC*-positive PDTC are not associated with increased aggressiveness or poor patient survival and usually show histological evidence indicating coexistence with or possible evolution from PTC often diagnosed as cPTC, SVPTC and tall cell PTC (69, 127, 133).

Concerning UTC, all previous studies reported an absence of *RET/PTC* rearrangements in this setting (69, 107); only the study by Mochizuki *et al.*, who studied seven composite UTC (UTC having a PTC component) and 14 single component UTC, has found the presence of a *RET/PTC3* rearrangement in both components (UTC and PTC) of one composite UTC, whereas all 14 single component UTC were *RET/PTC* negative (134).

PAX8-PPARɣ rearrangement

PAX8 is a transcription factor important for thyroid development, and in the mature gland it drives the expression of many thyroid-specific genes such as those encoding thyroglobulin, thyroid peroxidase and *NIS* (135). The peroxisome proliferator-activated receptor gamma (*PPARɣ*), member of the steroid/thyroid nuclear receptor family, is a

nuclear receptor/transcription factor essential for adipogenesis that is expressed at very low levels in the normal thyroid and has no known function in this organ (136).

The *PAX8-PPARɣ* fusion oncogene results from a balanced translocation, t(2;3)(q13:p25), that results in the fusion of the promoter and most of the *PAX8* gene to the coding exons of the *PPARɣ* gene. Thus the PAX8-PPARɣ fusion protein (PPFP) is expressed under the control of the *PAX8* promoter, which is highly active in the thyroid (137). The functional consequences of expression of PAX8-PPARɣ are still not fully understood (138).

A separate fusion protein resulting from a t(3;7)(p25;q34) chromosomal rearrangement between *CREB3L2* and *PPARɣ* has been reported in one case of FTC (139). That two distinct chromosomal translocations involving *PPARɣ* have been associated with FTC, suggests that modulation of PPARɣ-regulated pathways is important for *PAX8-PPARɣ*-mediated carcinogenesis. However the oncogenic mechanism of *PAX8-PPARɣ* is poorly understood and its functional relationship to *PPARɣ* is complex (23).

The prognostic significance of *PAX8-PPARɣ* expression in FTC or FVPTC has not been extensively studied. *PAX8-PPARɣ* is associated with a younger age at presentation and increased vascular invasion (140-142). However, others report that *PAX8-PPARɣ* is associated with indicators of good prognosis including markers of differentiation and few metastases (143).

An important point that remains to be elucidated is whether *PAX8-PPARɣ* is sufficient by itself to promote thyroid tumourigenesis or whether additional genetic or epigenetic events are required to enable the full phenotypic expression of follicular thyroid carcinoma. An average of different studies conducted so far shows the presence of *PAX8-PPARɣ* in 36% FTCs (0-63%), 11% of FTAs (0-55%), 13% of FVPTC (0-50%), but not in UTCs (144, 145).

In the majority of the FTA cases that harbour the *PAX8-PPARɣ* rearrangement, the nodule harbour a thick capsule suggesting that they may represent pre-invasive follicular carcinomas or malignant tumours where invasion was overlooked during histological examination(142).

PAX8-PPARɣ rearrangements are absent from all PDTC and UTC so far analysed (69, 87). It should also be pointed out that the data suggesting that *RET/PTC and PAX8-PPARɣ* are not found in UTCs or in higher percentages in DTCs and PDTCs is mostly performed through mRNA expression data and this is dependent on promoters of genes that are expressed in differentiated thyroid cells. In these cases, DNA studies, able to detect the presence of the rearrangement even in the absence of mRNA expression, are required to elucidate this controversial issue (146).

TERT promoter mutations

The thyroid tissue is a conditional-renewal tissue, which proliferates very slowly and rarely; human thyroid cells are supposed to divide about five times in the adult life (147).In the thyroid gland there is not a well-defined stem cell population that might constitute a pool of cells responsible for retaining the capacity of division. Some authors advanced that, the so-called Solid Cell Nests (SCNs) of the thyroid, which are embryonic remnants of the ultimobranchial body may represent the pool of the thyroid stem cells as they expressed several stem cell markers, namely telomerase (148).

Telomerase activation is known to be a hallmark of cancer (149), being detected in 80 to 90% of malignant tumours (150, 151). High telomerase activity has been reported in thyroid tumours, particularly in advanced forms of the disease, but is rare in normal thyroid tissues (152-154).

Human telomerase reverse transcriptase (*hTERT*) gene is located on chromosome band 5p15.33 and encodes the catalytic subunit of telomerase that together with a RNA component, TERC, maintains genomic integrity by telomere elongation (155). Though *TERT* and *TERC* are sufficient for *in vitro* telomerase activity, the *in vivo* telomerase functioning requires additional components that associate with *TERT* and *TERC*, to form the holoenzyme (156, 157).

The mechanism of *TERT* upregulation in cancers has been attributed to several mechanisms including epigenetic deregulation as well as genetic amplification of the locus containing *TERT* gene (158, 159). The recently discovered *TERT* promoter mutations add new dimensions to the acquisition of telomerase activity in human cancers, since somatic mutation in the coding region of *TERT* are infrequent in human cancer (160-163).

TERT promoter mutations occur in two hotspot positions, - 124 and - 146, where -1 is the base just upstream the A of the ATG translation start site. They represent nucleotide changes of -124 C>T and -146 C>T (G>A on opposite strand). Both mutations create an 11-base nucleotide stretch 5'-CCCCTTCCGGGG-3', which contains a consensus binding site, GGAA (in reverse complement), for ETS transcription factors, suggesting potentially important biological relevance for these mutations (160, 161).

In fact, the two mutations have been demonstrated to confer increased transcriptional activity on the *TERT* promoter (160, 161). These mutations are not found in normal human subjects and in the public genetic databases and are, therefore, cancer-specific somatic genetic alterations, further supporting their important role in human tumourigenesis. This is consistent with the previously observed increased telomerase activities in several human cancers (164, 165). Thus, *TERT* promoter mutations, by promoting the expression of the catalytic subunit of telomerase may play an important role in human carcinogenesis.

In thyroid cancer, recent studies observed that *TERT* promoter mutations were only found in TFC-derived cancer, such as DTC, PDTC and UTC. No mutations were described in normal adjacent thyroid tissue nor in benign lesions such as goiter, adenomas or thyroiditis (45, 163, 166).

Telomerase activity has been found in 48% of PTC, 71% of FTC and 78% of UTC (153, 167-169), whereas *TERT* promoter mutations were found in 12% of the DTC tumours (170) , being present in 7.5% of the PTCs (163, 170, 171), 17.1% of the FTC (163, 170), 29.0% of PDTC and 33.0% of UTCs (163, 170, 171), where the most frequent mutation was -124C>T mutation (163).

It has been suggested that telomerase may contribute to a more aggressive behaviour of the thyroid cancer (152, 168, 169), and *TERT* promoter mutations were associated in PTC with patient older age, larger tumour size and higher stage (170-173).

In PTC, a significant association between *BRAF* or *NRAS* and *TERT* promoter mutations was found, however this co-association was not associated with more aggressive clinico-pathological features or worse outcome than the cases that harboured just the *TERT* promoter mutation (173).

Until recently, *TERT* promoter mutations have only been identified in malignant thyroid lesions (163, 170, 173), however, in one cohort (174), it was found in a FTA a *TERT* promoter mutation -124C>T, and in two cases diagnosed as atypical FTA, an entity with uncertain malignant potential, with a frequency of 17%, which lead to the question at what stage of carcinogenesis occurs the *TERT* promoter mutation and telomerase activity (174).

TERT promoter mutations may play a role in the de-differentiation, progression, aggressiveness and may be one of the mechanisms that underlies telomerase reactivation in several types of human tumours (45).

Aims

The initial challenge for this work was the organization of a repository of thyroid tumours, providing an easier availability to DNA, RNA, and protein components with high quality, and with the main clinico-pathological and molecular data available for each case, to be used for furthers studies in the research group.

To achieve this goal, the first step was the organization and elaboration of a database of the frozen tissues from the repository. To construct the database it was necessary to select the cases according to the available histological report, and the clinico-pathological information that would be relevant to further studies.

The second aim was the development of a standard protocol allowing the extraction of DNA, RNA and protein lysates from the tumour sample. The purpose of the protocol was the analysis of the same area of the tumour, allowing a perfect match between the DNA, RNA and protein characteristics, insuring high quality and preservation of the extracted components.

The third aim was the characterization of the main genetic alterations in thyroid tumours from the repository – *BRAF* and *NRAS* mutations, *TERT* promoter mutations, and *RET/PTC1*, *RET/PTC3* and *PAX8-PPARγ* rearrangements.

Having the genetic characterization available and to access the quality and representation of the repository, statistical analyses were performed to verify the clinico-pathological and genetic associations.

Materials and Methods

Samples

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

The frozen samples were obtained from CHSJ, between the years 1989 and 2012, and kept at a temperature of -80°C at the Institute of Molecular Pathology and Immunology of the University of Porto (Ipatimup). Diagnosis and clinico-pathological data were retrieved from the files of the Department of Pathology from the CHSJ.

The repository consisted of 500 cases. According to the availability of pathological report or clinical information 225 samples from 184 patients were selected for the subsequent study. Those samples correspond to 54 samples of nodular goiter, 24 samples of FTA, 117 samples of malignant tumours and 30 samples of normal thyroid.

DNA, RNA and protein extraction

Sample preparation

The samples were thawed at room temperature and fragments representative of the entire sample were taken, each with a dimension of about 1 cm. The fragments were homogenized in 2mL TRIzol® Reagent (ref 15596018, Life Technologies™, Carlsbad, USA). After tissue homogenization, the sample was equally divided in two tubes (1.5 mL), for DNA / RNA extraction and protein extraction. The sample was then stored at -80ºC.

RNA extraction

The RNA extraction was performed according to the manufacturer protocol (175), where the tube with the sample for DNA/RNA extraction was incubated at room temperature for 5 minutes to allow the complete dissociation of the nucleoprotein complex.

With the purpose of separating the RNA, DNA and proteins, it was induced the chloroform phase separation by the addition of 200µL chloroform to the sample, and vigorously mixed and incubated 2-3 minutes at room temperature. The sample was then centrifuged at 12,000g for 15 minutes at 4ºC and the aqueous phase was carefully removed to a new tube (1.5 mL) and added 500 µL absolute isopropanol, and the remaining phases (interphase and organic) were stored at -80º for DNA extraction.

The tube with RNA and isopropanol was inverted several times and incubated at 4ºC for 10 minutes. Subsequently the sample was centrifuged at 12,000g for 10 minutes at 4ºC. The supernatant was removed and discarded; the RNA pellet was washed in 1mL 75% ethanol and then centrifuged at 12,000g for 5 minutes at 4ºC. Again the supernatant was removed and discarded; the RNA was dried for a few seconds in a hot plate at 55ºC.

The RNA pellet was then dissolved in 50µL of DNase and RNase free water and quantified through the Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, Lithuania, EU). The samples were then stored at -80ºC.

DNA extraction

The tube (1.5mL) that contained the interphase and organic phase was vigorously mixed, centrifuged at 12,000g for 15 minutes at 4ºC, and the possible remaining aqueous phase removed and discarded.

Absolute ethanol (300µL) was added to precipitate the DNA, and the sample was mixed by inversion several times being thereafter incubated at room temperature for 2-3 minutes. The sample was then centrifuged at 12,000g for 10 minutes at 4ºC. The supernatant was removed and discarded; the pellet was dried for a few seconds in a hot plate at 55ºC.

Lysis Solution (600µL; ref CL-250, Citogene®, Citomed, Lisbon, Portugal) was added to the sample and vortexed. Subsequently, 20µL Proteinase K (20mg/dL) was added to the mixture, and incubated overnight at 55ºC and with shaking (125rpm). The samples were incubated with more 10µL Proteinase K 20mg/dl for more 4-5 hours in the same conditions previously described.

The sample was cooled at room temperature, and 200µL of Protein Precipitation Solution (ref PP-125, Citogene®, Citomed, Lisbon, Portugal) was added and mixed for 20 seconds, and incubated in ice for at least for 5 minutes. Then, the samples were centrifuged at 16,000g for 3 minutes at 0ºC.

The supernatant was transferred to a new tube (1.5mL) containing 600µL of absolute isopropanol and 1µL of glycogen (20mg/mL; ref #50561, Thermo Scientific, Lithuania, EU), and the mixture was inverted at least 50 times. Then the tube was centrifuged at 16,000g for 3 minutes at 15ºC. The supernatant was removed and discarded; the DNA pellet was washed in 300µL of ethanol (70%). The tube was inverted several times, and centrifuged at 16,000g for 3 minutes at 15ºC.

The supernatant was carefully removed and discarded, and the pellet was dried in a hot plate at 55ºC for 10 seconds. Then the DNA pellet was dissolved in 50µL of DNase and RNase free water, quantified by Nanodrop N-1000 Spectrophotometer (Thermo Scientific, Lithuania, EU) and stored at -20ºC.

Protein extraction

The protein extraction was done according to the modified TRIzol® protocol developed by Simões *et al.* (176). In order to promote the phase separation, as previously explained, 200 µL of chloroform was added to the sample, which was incubated at room temperature for 3 minutes and centrifuged at 12,000 g for 15 minutes at a temperature of

4ºC, where the aqueous phase was removed and discarded. The centrifugation step was repeated at least once to remove the remaining aqueous phase supernatant.

To precipitate the DNA, the samples were mixed by inversion of the tube with 300µL absolute ethanol and then centrifuged at 2,000g for 5 minutes at 4 ºC.

The supernatant was removed to a 2mL tube, where 1.5mL isopropanol was added to precipitate the proteins. The sample was mixed and incubated at room temperature for 10 minutes. Subsequently the sample was centrifuged at 12,000g for 10 minutes at 4ºC and the supernatant discarded.

Proteins pellets were next washed three times in 2mL 0.3M guanidine hydrochloride in 95% ethanol. In each wash, the sample was vigorously mixed, incubated at room temperature for 20 minutes and centrifuged at 7,500g for 5 min at 4ºC.

After the final wash and spin, 2mL absolute ethanol was added; the sample was incubated at room temperature for 20 minutes, and centrifuged at 7,500g for 5 minutes at 4ºC.

The supernatant was removed and 1mL 1:1 solution of 1% SDS and 8M urea in Tris-HCl 1M, pH 8.0 was added to the protein pellets, followed by 5 cycles of 20 seconds sonication and 30 seconds of ice incubation (Bandelin Sonopuls, model HD2070, Heinrichstraβe, Berlin - 70 watts, ultrasonic frequency 20kHz), to solubilize the protein.

Finally, the sample was centrifuged at 3,200g for 10 min at 4ºC, to sediment insoluble material. The supernatant containing the solubilized proteins was transferred to a new 1.5 ml tube and stored at -80ºC.

cDNA synthesis

With the purpose to verify the quality of the extracted RNA and to study the rearrangements, cDNA for each sample was synthetized. To guarantee the preparation of DNA-free RNA, the samples were treated with DNase I, RNase-free, which is an endonuclease that cleaves DNA in a non-specifically way to release 5'-phosphorylated di-, tri-, and oligonucleotide products (177).

To this procedure 1µg of RNA from each sample was diluted in DNase and RNase free water, in 8µL of total volume and 1µL of DNase I RNase-free 1U/µl and 1µL of 10X Reaction Buffer with MgCl2 for DNase I were added, followed by incubation at 37°C for 30 minutes. After the period of incubation 1µL of 50 mM EDTA was added to the mix followed by incubation at 65ºC for 10 minutes (ref #EN0521, Thermo Scientific, Lithuania, EU).

To this mix 1 µL of Random Hexamer Primer 0.2 µg/µL (ref #SO142, Thermo Scientific, Lithuania, EU) was added, and incubated at 65ºC for 5 minutes. After this step the sample was immediately put on ice. The Random Hexamer Primers are a mixture of single-stranded random hexanucleotides with 5'- and 3' hydroxyl ends.

A RT master mix, in a total of 8µL, was prepared with 0.5µL of Ribolock RNase Inhibitor 40U/µL (ref #EO0382, Thermo Scientific, Lithuania, EU), a component that inhibits the activity of the RNases A, B and C by binding them in a non-competitive mode at 1:1 ratio; 4µL of 5X Reaction Buffer for reverse transcriptase, 2µL of dNTP Mix 40mM (10mM each, ref #RO192, Thermo Scientific, Lithuania, EU), 0.5µL of DNase and RNase free water, and 1µL of RevertAid Reverse Transcriptase 200U/µL(ref #EP0441, Thermo Scientific, Lithuania, EU). The master mix was added to the previous prepared mix and followed incubation at 25ºC for 10 minutes, 42ºC for 60 minutes and 70ºC for 10 minutes.

To the final product 20µL DNase and RNase free water was added, finalizing the procedure with 40µL of cDNA.

Table 1 - *Number of cases extracted, with the description of the samples of each cases and procedure applied in each one.*

Number of Cases	Sample	Procedure
54 Benign lesions (Nodular Goiter)	Lesion	Homogenized and stored in TRIzol at -80˚C;
20 Benign lesions (Follicular Adenoma)	Lesion	DNA, RNA and Protein extraction and genetic alterations analysis
2 Benign lesions (Follicular Adenoma)	Lesion and normal thyroid tissue	
1 Benign lesion (Follicular Adenoma)	Two independent lesions and normal thyroid tissue	
74 Malignant lesions	Lesion	
23 Malignant lesions	Lesion and normal thyroid tissue	
6 Malignant lesions	Two independent lesions	
4 Malignant lesions	Two independent lesions and normal thyroid tissue	

Genetic alterations

The tumours were characterized for the most frequent genetic alterations in thyroid tumours, namely *BRAF*, *NRAS* and *TERT* mutations. The presence of *RET/PTC1*, *RET/PTC3* and *PAX8-PPARɣ* rearrangements were also screened.

Polymerase Chain Reaction (PCR) was performed with GoTaq® G2 Flexi DNA Polymerase (Promega, WI, USA), for the detection in the hotspot regions of *NRAS* (codon 61), *BRAF* (Exon 15) and *TERT* (promoter region) mutations, under the conditions described by Castro *et al.* (144) and Vinagre*, et al. (163)*.

For *BRAF* and *NRAS* the PCR final mix, 25µL in total, contained 100ng of genomic DNA, 1µL of 4you4 dNTP Mix 10mM each (ref 110001,BIORON GmbH, Ludwigshafen, Germany), 1µL of each primer 10mM (forward and reverse), 5µL of 5X Green GoTaq® Flexi Buffer (ref M891A, Promega, Madison, WI, USA), 0.15µL of GoTaq® G2 Flexi DNA Polymerase 1U/µL (ref M830B, Promega, Madison, WI, USA), 13.35µL of DNase and RNase free water and 2.5µL of $MgCl_2$ 25mM (ref A315H, Promega, Madison, WI, USA).

For *TERT* the PCR final mix, 20µL in total, contained 100ng of genomic DNA, 4µL of 5X Green GoTaq® Flexi Buffer, 1.2µL de MgCl2 25mM, 0.8µL 4you4 dNTP Mix 10mM each, 0.45µL of each primer 10mM (forward and reverse), 12µL of DNase and RNase free water and 0.1 µL of GoTaq® G2 Flexi DNA Polymerase 1U/µL.

The presence of the rearrangements was determined through Reverse Transcriptase PCR (RT-PCR), using GoTaq® G2 Flexi DNA Polymerase (Promega, WI, USA). The cDNA sequences were analysed for the *PAX8-PPARɣ* rearrangement according to the procedure described by Marques, *et al.* (178), *RET/PTC1* and *RET/PTC3* according to Lima, *et al.* (179). To access the quality of the synthetized cDNA, previously described, a RT-PCR was performed for a house-keeping gene, the *β-actin* gene.

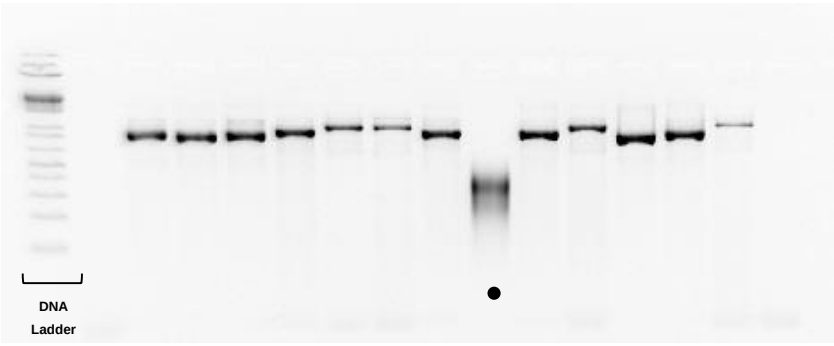

Figure 3 - *Image representative of the result of an electrophoresis gel of the RT- PCR products for the β-actin gene, with a size of about 877 base pairs, captured by ChemiDoc™ XRS Imaging System. The signalled sample has degraded RNA, which result in an inefficient amplification.*

RT-PCR was performed, with a final mix of 25µL. It contained 2µL of cDNA, 5µL of 5X Green GoTap® Flexi Buffer; 1.5µL of MgCl2 25mM; 1µL 4you4 dNTP Mix 10mM each;

1µL of each primer 10mM (forward and reverse), 13.2µL of DNase and RNase free water and 0.3µL of GoTaq® G2 Flexi DNA Polymerase 1U/µL.

All the RT-PCR reactions were performed using samples positive for each rearrangement in question, to assure the efficiency of the reaction.

All the PCR and RT-PCR reactions were run in GeneAmp® PCR System 2700 (Applied Biosystems®, CA, USA), with an annealing temperature of 55°C for *RET/PTC1* and *RET/PTC3*; 58°C for *BRAF*, *PAX8-PPARγ* and *β-actin*; 57°C for *NRAS* and 62°C for *TERT*.

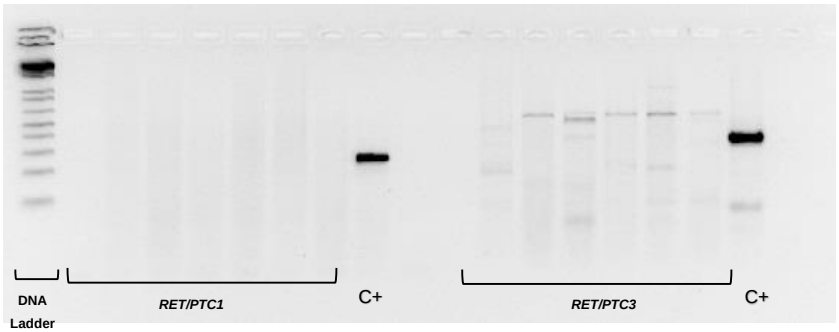

Figure 4 - *Image representative of the result of an electrophoresis gel for the RT-PCR reaction for the RET/PTC1, with a size of about 200 base pairs, and RET/PTC3 rearrangements, with a size of about 387 base pairs, respectively, captured by ChemiDoc™ XRS Imaging System, with the cases screened for the rearrangement and the positive control signalled. The C+ represents the positive control for each PCR reaction; all the cases analysed in the gel were negative for the rearrangement.*

To insure the efficiency of the PCR and RT-PCR reactions the products were run in a 2% agarose gel electrophoresis, using the SGTB 1x buffer (ref GB01.0520, GRiSP, Oporto, Portugal). The samples were mixed with 1µL of Loading Buffer with Gel Red® Nucleic Acid Gel Stain 3X (ref 41003, Biotium, Inc., CA, USA), where the Loading Buffer provides density to the sample and include coloured 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 (Biotium Inc., Ca, USA).To evaluate the size of the PCR products in the electrophoresis gel, 1kb Plus DNA Ladder (ref 10787-026, Invitrogen, CA, 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) (Figure 3).

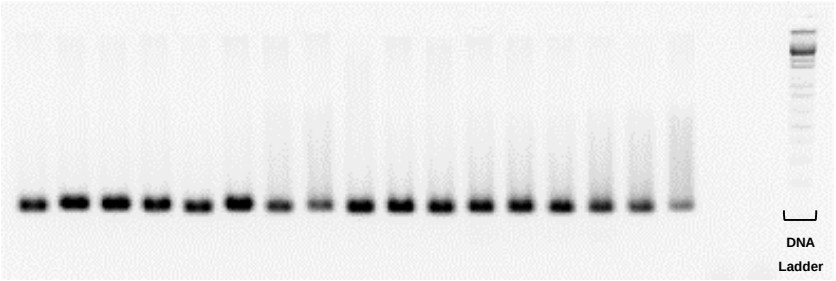

Figure 5 - *Figure representative of the results of an agaroses gel electrophoresis for the PCR of NRAS codon 61, with a size of about 119 base pairs, captured with ChemiDoc™ XRS Imaging System, where all the cases studied showed amplification of the fragment of interest.*

To allow the sequencing of the PCR and RT-PCR products it was necessary to purify them. The ExoSAP method uses two hydrolytic enzymes, Exonuclease I 20U/µL (ref #ENO582, Thermo Scientific, Lithuania, EU) and Shrimp Alkaline Phosphatase (Fast AP Thermosensitive Alkaline Phosphatase 1U/µL, ref #EF0651, Thermo Scientifics, Lithuania, EU), to remove the unwanted extraneous single-stranded DNA, dNTPs and primers that were not used in the PCR reaction.

To the PCR product (10µL) 1.5 µL of ExoSAP was added, followed by incubation at 37ºC for 30 minutes, which was the optimal temperature for the enzymes action, and 80ºC for 15 minutes for their inactivation.

This procedure of purification was applied in all the PCR and RT-PCR products. However in some cases, in the RT-PCR reaction, it occurred the amplification of several bands. In those it was necessary to proceed to the extraction of bands in 1% agarose gel electrophoresis, to allow the sequencing of the samples. For this, it was used the DNA Gel Extraction Kit (LSKGEL050, Millipore, MA, USA), according to the manufacturer procedure.

All the PCR products for *BRAF*, *NRAS* and *TERT* promoter hotspot mutations, and the RT-PCR positive cases for rearrangements, were analysed by DNA sequencing (Sanger sequencing) using the ABI Prism BigDye Terminator Kit v3.1 Cycle Sequencing (ref 4337455, Applied Biosystems®, Warrington, UK).

The final sequencing mix, total 10µL, has incorporated 0.5µL of Big Dye, 3,5µL of 5X Sequence buffer (Big Dye® Terminator v1.1, v1.3, ref 4336697, Applied Biosystems®, Warrington, UK), 0.3µL of the primer of interest 10mM (forward or reverse), 2.7µL DNase and RNase free water and 3µL of the purified PCR product. The final mix was amplified in a GeneAmp® PCR System 2700 (Applied Biosystems®, CA, USA) termocycler, with an annealing temperature of 55 ºC.

The final amplified product was precipitated in Zetadex- 50 Superfine Gel Filtration Matrix (ref TM-0104-E500.0-001, emp Biotech GmbH, Berlin, Germany) columns to remove all the ddNTPs that were not incorporated and that could interfere with the reading signal conducted by the laser.

After precipitation, 15µL of HiDi™ Formamide (ref 1403305, Applied Byosystems®, Woolsten Warrington, UK) was added to the sample. Finally the sample was analysed in the ABI prism 3100 Genetic Analyzer (Perkin-Elmer).

All positive cases for mutations and rearrangements were validated by a new independent analysis.

Statistical analysis

Statistical analysis was conducted with IBM SPSS Statistics version 21 (IBM, New York, USA). The results were expressed in frequency, percentage and mean, taking into count that there were 135 independent lesions that belonged to 128 patients.

For the analysis of the relationship between patient's age and tumour size with diagnosis, histological characteristics and molecular status, unpaired t-test, Mann-Whitney test and analysis of variance was used.

Chi-Square with Fisher's correction was used in the statistical analysis of the other parameters, which included diagnosis, histological and clinical characteristics and molecular status.

Results

The construction of the database with the clinico-pathological data and genetic characterization was successful. The development of standard protocols for the extraction of nucleic acids and proteins of high quality was complete with high success rates.

Of the 171 extracted samples (Table 1), tumours and normal thyroids, a success rate of 99.4% for DNA extraction was achieved, where only the extraction of one sample of a malignant tumour was inefficiently obtained.

In the RNA extraction, an efficiency rate of 97.7% was obtained, where the extraction of RNA was unsuccessful for 3 tumours samples, two malignant and one benign, and for one normal thyroid sample. The sample, from which DNA extraction was proved as inefficient, was also one of the 3 samples whose RNA extraction was also ineffective.

The clinico-pathological characterization for the database, which was performed by obtaining information from histopathological reports, remained incomplete in some cases. Due to the update in recent years in the criteria for diagnosis, many of the considered fields were not mentioned in the report and remained unfulfilled. This limitation of the study was considered in the construction of the database and also in the statistical analysis performed.

The samples included in the database belonged to 128 patients, whose mean age was 44 (11-82) years. Of these patients 22 were male (17.5%), with mean age of 41 (11-80) years. There were 104 female patients (82.5%), with a mean age of 45 (13-82) years.

Table 2 presents the frequency of diagnosis of the 135 tumour samples belonging to 128 patients, where were considered 7 patients who harboured 2 independent tumours.

Table 2 *– Diagnosis of the extracted tumour samples that composed the repository (n=135).*

Diagnosis	Number	Percent (%)
FTA	24	17.8
FTC	14	10.4
PTC	63	46.7
FVPTC	25	18.5
PDTC	3	2.2
MTC	3	2.2
Metastasis	3	2.2
Total	135	100.0

Legend: FTA, follicular thyroid adenoma; FTC, follicular thyroid carcinoma; PTC, papillary thyroid carcinoma; FVPTC, follicular variant papillary thyroid carcinoma; PDTC, poorly differentiated thyroid carcinoma.

Table 3 - *Characterization of the genetic alterations in the tumour samples (n=135).*

Genetic alterations		Tumour diagnosis							
		FTA	**FTC**	**PTC**	**FVPTC**	**PDTC**	**MTC**	**DM**	**Total**
BRAF	**WT**	**12** 100.0%	**14** 100.0%	**39** 61.9%	**23** 92.0%	**3** 100.0%	**3** 100.0%	**3** 100.0%	**97** 78.9%
	p.V600E	**0** 0.0%	**0** 0.0%	**24** 38.1%	**1** 4.0%	**0** 0.0%	**0** 0.0%	**0** 0.0%	**25** 20.3%
	p.K601E	**0** 0.0%	**0** 0.0%	**0** 0.0%	**1** 4.0%	**0** 0.0%	**0** 0.0%	**0** 0.0%	**1** 0.8%
NRAS	**WT**	**23** 95.8%	**10** 71.4%	**57** 90.5%	**19** 76.0%	**2** 66.7%	**2** 66.7%	**2** 66.7%	**115** 85.2%
	p.Q61R	**1** 4.2%	**4** 28.6%	**6** 9.5%	**4** 16.0%	**0** 0.0%	**0** 0.0%	**1** 33.3%	**16** 11.9%
	p.Q61K	**0** 0.0%	**0** 0.0%	**0** 0.0%	**2** 8.0%	**1** 33.3%	**1** 33.3%	**0** 0.0%	**4** 3.0%
TERT	**WT**	**23** 95.8%	**13** 92.9%	**61** 98.4%	**24** 96.0%	**3** 100.0%	**3** 100.0%	**2** 66.7%	**130** 96.3%
	-124 C>T	**1** 4.2%	**1** 7.1%	**0** 0.0%	**1** 4.0%	**0** 0.0%	**0** 0.0%	**0** 0.0%	**3** 2.2%
	-146 C>T	**0** 0.0%	**0** 0.0%	**0** 0.0%	**0** 0.0%	**0** 0.0%	**0** 0.0%	**1** 33.3%	**1** 0.7%
	-150 C>T*	**0** 0.0%	**0** 0.0%	**1** 1.6%	**0** 0.0%	**0** 0.0%	**0** 0.0%	**0** 0.0%	**1** 0.7%
RET/PTC1	**WT**	**23** 100.0%	**13** 92.9%	**53** 85.5%	**23** 95.8%	**3** 100.0%	**3** 100.0%	**2** 66.7%	**120** 90.9%
	Rearranged	**0** 0.0%	**1** 7.1%	**9** 14.5%	**1** 4.2%	**0** 0.0%	**0** 0.0%	**1** 33.3%	**12** 9.1%
RET/PTC3	**WT**	**23** 100.0%	**14** 100.0%	**61** 98.4%	**23** 95.8%	**3** 100.0%	**3** 100.0%	**3** 100.0%	**130** 98.5%
	Rearranged	**0** 0.0%	**0** 0.0%	**1** 1.6%	**1** 4.2%	**0** 0.0%	**0** 0.0%	**0** 0.0%	**2** 1.5%
PAX8-PPARɣ	**WT**	**23** 100.0%	**13** 92.9%	**62** 100.%	**23** 95.8%	**3** 100.0%	**3** 100.0%	**3** 100.0%	**130** 98.5%
	Rearranged	**0** 0.0%	**1** 7.1%	**0** 0.0%	**1** 4.2%	**0** 0.0%	**0** 0.0%	**0** 0.0%	**2** 1.5%

Legend: FTA, follicular thyroid adenoma; FTC, follicular thyroid carcinoma; PTC, papillary thyroid carcinoma; FVPTC, follicular variant papillary thyroid carcinoma; PDTC, poorly differentiated thyroid carcinoma; MTC, medullary thyroid carcinoma; DM, distant metastases; WT, wild-type; * TERT polymorphism.

Of the 135 tumours samples, 18% were diagnosed as FTA; 75% were DTCs (11% FTCs and 46% were PTCs); 2% were PDTC; 2% were distant metastases from primary tumours with PTC and FVPTC diagnosis; and 2% were MTC (Table 2).

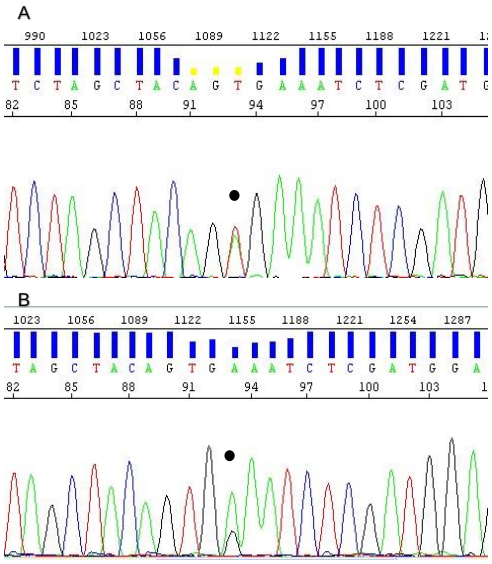

Figure 6 - *Representative result obtained through sequencing of BRAF exon 15, primer forward.* ***A:*** *Representative chromatogram of the signalled BRAF p.V600E mutation, thymine to adenine transversion, c.1799T>A;* ***B:*** *Representative chromatogram of the signalled BRAF p.K601 mutation, adenine to guanine transversion, c.1801A>G.*

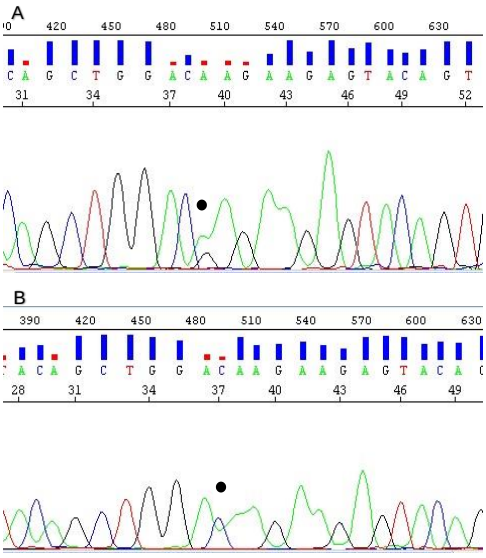

Figure 7 - *Representative result obtained through sequencing of NRAS codon 61 region, primer forward.* ***A:*** *Representative chromatogram of the signalled NRAS (p.Q61R) mutation, adenine to guanine transversion, c.182A>G;* ***B:*** *Representative chromatogram of the signalled NRAS (p.Q61K) mutation, cytosine to adenine transversion, c.181C>A.*

The FTA group comprised 18 samples of FTA, 4 samples of fetal FTA, and 1 sample of oncocytic FTA. The PTC group comprised 54 samples of cPTC; 5 samples of diffuse sclerosing PTC; 2 samples of tall cell PTC; 1 sample of SVPTC; and 1 sample of oncocytic PTC, whereas the FTC group comprised 13 samples of FTC and 1 sample of oncocytic FTC.

Table 3 presents the genetic alterations found in the tumour samples, grouped by diagnosis as previously described, and supplementary Tables 14 and 15 present the genetic alterations comprising all the diagnosis individually.

Considering all tumour samples analysed, 25 (20.3%) were positive for the *BRAF* (p.V600E) mutation (Figure 6A) and 1 (0.8%) tumour was positive for the *BRAF* (p.K601E) mutation (Figure 6B).

In the genetic analysis of *BRAF* exon 15, it was interesting to report that one particular patient harboured two nodules, both cPTC, where one of them harboured a *BRAF* (p.V600E) mutation whereas the other one was negative for *BRAF* mutations. In another patient with two cPTCs, both nodules harboured the *BRAF* (p.V600E) mutation.

When the *NRAS* point mutations in codon 61 were screened, 16 tumours (12%) had the *NRAS* (p.Q61R) mutation (Figure 7A) and 4 tumours (3%) had the *NRAS* (p.Q61K) mutation (Figure 7B).

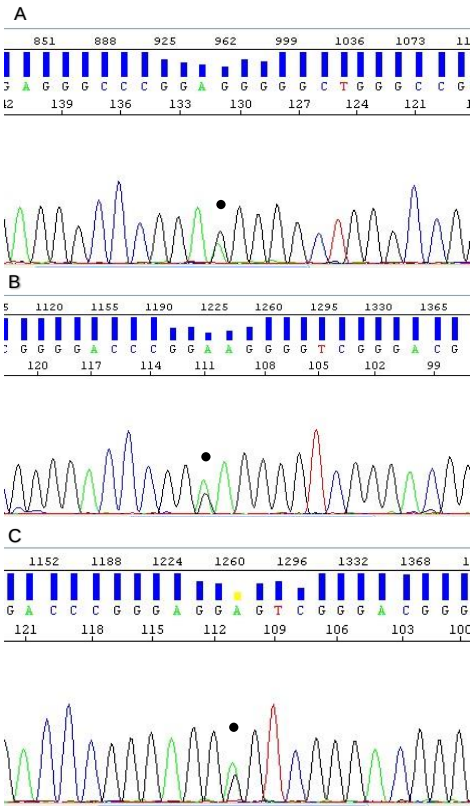

Figure 8 - *Representative result obtained through sequencing of the TERT promoter region, primer reverse.* ***A:*** *Representative chromatogram of the signalled -124C>T mutation, a cytosine to thymine transversion, (G>A in the opposite strand);* ***B:*** *Representative chromatogram of the signalled -146C>T mutation, cytosine to thymine transversion, (G>A in the opposite strand);* ***C:*** *Representative chromatogram of the signalled -150C>T mutation, cytosine to thymine transversion, (G>A in the opposite strand*

Within this genetic analysis it was curious to report that the majority of the FVPTCs positive for the *NRAS* mutations were encapsulated.

In the analysis of the promoter region of the *TERT* gene, 3 tumours (2%) harboured the -124C>T mutation (Figure 8A), 1 tumour (1%) harboured the -146C>T mutation (Figure 8B) and 1 tumour (1%) harboured the -150 C>T polymorphism (Figure 8C).

The PTC harbouring the *TERT* -150 C>T polymorphism, overlapped with a *BRAF* (p.V600E) mutation (Table 4).

One of the two samples of distant metastases, from a primary tumour diagnosed as FVPTC, harboured a *TERT* promoter -146 C>T mutation and a *NRAS* (p.Q61R) mutation (Table 4).

For *RET/PTC1* rearrangement, 12 tumours (9.1%) were positive (Figure 9A) and in *RET/PTC3* rearrangement screening 2 tumours (1.5%) were positive (Figure 9B).

Table 4 *– Malignant tumour samples with overlapping of genetic alterations.*

Tumour diagnosis	Age (years)	Tumour size (cm)	Overlapping of genetic alterations
cPTC	22	1.4	***TERT* -150C>T*** ***BRAF* (p.V600E)**
FVPTC metastasis	63	7.5	***TERT* – 146C>T)** ***NRAS* (p.Q61R**
cPTC	41	4.5	***BRAF* (p.V600E)** ***RET/PTC1***
FTC minimally invasive	56	4.0	***RET/PTC1*** ***PAX8-PPARɣ***

Legend: cPTC, classic papillary thyroid carcinoma; FVPTC, follicular variant of papillary thyroid carcinoma; FTC, follicular thyroid carcinoma; * *TERT* polymorphism.

In one case of PTC where tissue from the primary and corresponding metastases was available, both samples were positive for *RET/PTC1* rearrangement (Table 3).

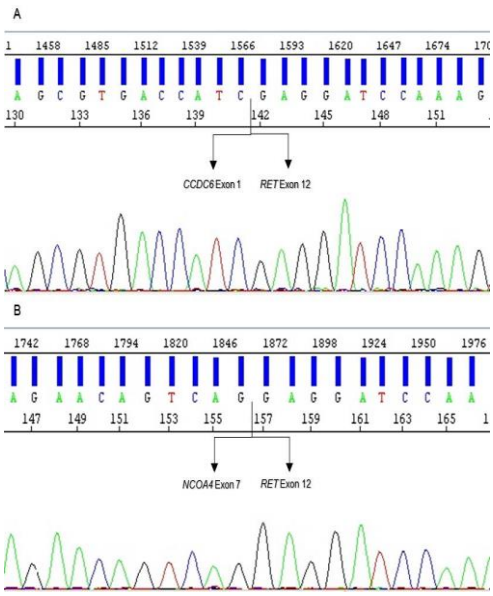

Figure 9 - *Representative result obtained through sequencing of the RT-PCR for RET/PTC rearrangements.* ***A:*** *Representative chromatogram of the signalled fusion between the exon 1 of CCDC6 gene with the exon 12 of the RET gene, primer forward.* ***B:*** *Representative chromatogram of the signalled fusion between the exon 7 of NCOA4 gene and the exon 12 of RET gene.*

A PTC harboured the overlapping of a *BRAF* (p.V600E) mutation and *RET/PTC1* rearrangement (Table 4).

Two tumours (1.5%), a FTC and a FVPTC, were positive for the *PAX8-PPARɣ* rearrangement, (Figure 10). The FTC positive for *PAX8-PPARɣ* rearrangement was also positive for the *RET/PTC1* rearrangement (Table4).

For the statistical analysis only the tumours diagnosed as FTC, PTC and FVPTC were selected, given their malignant status, significant number of samples, the complete genetic characterization and their pathological reports, which were the most complete. We excluded from the analysis all benign lesions, distant metastases, PDTC, MTC, while the rare variants, as diffuse sclerosing PTC, tall cell PTC, SVPTC and oncocytic PTC were included in the PTC group; whereas the oncocytic FTC was included in the FTC group, as previously mentioned.

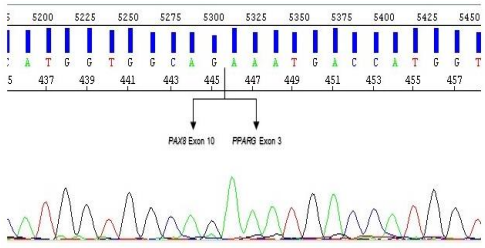

Figure 10 - *Representative result obtained though sequencing of the RT-PCR product for PAX8-PPARɣ rearrangement, primer forward, with the representative chromatogram of the fusion between exon 10 of PAX8 gene and exon 3 of PPARɣ gene.*

The number of tumours considered was 102, where the mean age of the patients was 41 (11-82) years. From the selected group 14 were male patients (14.1%) and 83 were female patients (85.9%).

Considering only the three diagnosis, 63 tumours were PTC (61.8%), 25 were FVPTC (24.5%) and 14 tumours were FTC (13.7%).

Table 5 presents the clinico-pathological characterization collected from these samples.

Considering the three groups, 25 tumours (24.5%) harboured the *BRAF* (p.V600E) mutation and 1 tumour (1.0%) harboured the *BRAF* (p.K601E) mutation. For the *NRAS* analysis 14 samples (13.7%) harboured the *NRAS* (p.Q61R) mutation and 2 tumours (2.0%) the *NRAS* (p.Q61K) mutation. In the *TERT* promoter 2 tumours (2.0%) had the *TERT* -124 C>T mutation.

When the rearrangements were analysed, 11 tumours (11.0%) were positive for the *RET/PTC1* rearrangement, 2 tumours (2.0%) were positive for *RET/PTC3* rearrangement and 2 tumours (2.0%) were positive for the *PAX8-PPARɣ* rearrangement.

Table 5 *– Clinico-pathological characterization of the malignant tumour samples.*

Characteristics (n)		PTC	FVPTC	FTC
Frequency (102)		**63** 61.8%	**25** 24.5%	**14** 13.7%
Gender (101)	**Male (14)**	**7** 11,3%	**4** 16,0%	**3** 21,4%
	Female (83)	**55** 88,7%	**21** 84,0%	**11** 78,6%
Age (101)	**Mean (range)**	**39.2** (11-76)	**43.0** (16-70)	**51.9** (29-82)
Tumour size (101)	**Mean (cm)**	**2.7** (0.20-10.00)	**2.1** **(**0.30-5.50)	**3.7** (1.40-7.00)
Number of tumours (102)	**Single**	**33** 52.4%	**16** 64.0%	**7** 50.0%
	Multiple	**30** 47.6%	**9** 36.0%	**7** 50.0%
Tumour capsule (101)		**22** 35.5%	**13** 52.0%	**14** 100.0%
Tumour capsule invasion (41)		**10** 66.7%	**2** 16.7%	**14** 100.0%
Lymph node metastasis (74)		**19** 44.2%	**4** 21.1%	**0** 0.0%
Vascular invasion (101)		**33** 53.2%	**4** 16.0%	**10** 71.4%
Extra-thyroid invasion (89)		**19** 36.5%	**2** 8.7%	**0** 0.0%
Hashimoto thyroiditis (79)		**3** 6.3%	**0** 0.0%	**0** 0.0%
Lymphocytic thyroiditis (102)		**28** 44.4%	**8** 32.0%	**4** 28.6%
Multinodular goiter (101)		**12** 19.4%	**7** 28.0%	**4** 28.6%
Other lesions (71)		**34** 69.4%	**11** 61.1%	**1** 25.0%
Minimally invasive (14)*		-	-	**10** 71.4%
Widely invasive (14)*		-	-	**4** 28.6%

Legend: PTC, papillary thyroid carcinoma, FVPTC, follicular variant of papillary thyroid carcinoma, FTC, follicular thyroid carcinoma; min, minimum age; max, maximum age; * only for FTC.

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When the three groups were compared, several statistical differences and associations were found. Although some associations have no immediate biological significance, we decided to show them as results found in the statistical analysis. The

following tables present the most relevant results that were statistically significant or presented a tendency, for each variable analysed.

The PTC group and the FVPTC groups harboured several differences (Table 6), where the patients within the PTC group harboured more lymph node metastasis (42.2% *vs.* 21.1%), vascular invasion (53.2% *vs.* 16.0%) and extrathyroidal invasion (36.5% *vs.* 8.7%) when compared with the FVPTC group.

PTC group harboured more frequently *BRAF* mutations (38.1% *vs.* 8.0%) than the FVPTC group; however the PTC group had less *NRAS* mutations (9.5% *vs.* 24.0%) than the FVPTC group.

Table 6 *– Comparison between the PTC and FVPTC, in relation to the variables lymph node metastasis, vascular invasion, extra-thyroid invasion, BRAF and NRAS mutational status.*

Characteristics		**Tumour diagnosis**		**p-value**
		PTC	**FVPTC**	
Lymph node metastasis	**Absent**	**24** 55.8%	**15** 78.9%	**0.071**
	Present	**19** 44.2%	**4** 21.1%	
Vascular invasion	**Absent**	**29** 46.8%	**21** 84.0%	**0.001**
	Present	**33** 53.2%	**4** 16.0%	
Extra-thyroid Invasion	**Absent**	**33** 63.5%	**21** 91.3%	**0.013**
	Present	**19** 36.5%	**2** 8.7%	
BRAF	**WT**	**39** 61.9%	**23** 92.0%	**0.005**
	Mutated	**24** 38.1%	**2** 8.0%	
NRAS	**WT**	**57** 90.5%	**19** 76.0%	**0.079**
	Mutated	**6** 9.5%	**6** 24.0%	

Legend: Statistical differences are considered as significant when p-value<0.05; PTC, papillary thyroid carcinoma, FVPTC, follicular variant of papillary thyroid carcinoma; WT, wild type.

When the FVPTC and FTC groups were compared (Table 7), it was possible to observe that the patients with FTC diagnosis harboured larger tumours (3.7 cm *vs*. 2.1 cm). All the FTCs were encapsulated and all of them presented tumours capsule invasion, whereas in the FVPTC group, 52.0% of the tumours were encapsulated and only 16.7% of them had tumour capsule invasion. The FTC group also harboured more frequently vascular invasion (71.4% *vs.* 16.0%) than the FVPTC.

Table 7 *- Comparison between the FVPTC and FTC, in relation to the variables tumour size, tumour capsule, tumour capsule invasion and vascular invasion.*

Characteristics		Tumour diagnosis		p-value
		FVPTC	FTC	
Tumour size	**Mean (cm)**	**2.08**	**3.68**	**0.003**
Tumour capsule	**Absent**	**12** 48.0%	**0** 0.0%	**0.001**
	Present	**13** 52.0%	**14** 100.0%	
Tumour capsule invasion	**Absent**	**10** 83.3%	**0** 0.0%	**0.000**
	Present	**2** 16.7%	**14** 100.0%	
Vascular invasion	**Absent**	**21** 84.0%	**4** 28.6%	**0.001**
	Present	**4** 16.0%	**10** 71.4%	

Legend: Statistical differences are considered as significant when p-value <0.05; FVPTC, follicular variant of papillary thyroid carcinoma; FTC, follicular thyroid carcinoma.

When the analysis between the PTC and FTC group was performed (Table 8), it was possible to report that the patients within the PTC group were younger (39 years *vs.* 52 years) and had smaller tumours (2.7cm *vs.* 3.7cm) when compared with the FTC group. All the FTCs were encapsulated and all of them presented tumour capsule invasion, whereas in the PTC group, 35.5% of the tumours were encapsulated and 66.7% of them had tumour capsule invasion.

The PTC patient's harboured more frequently lymph node metastasis (44.2%) and extra-thyroid invasion (36.5%), when compared with the FTC group, where none of the patients harboured these characteristics.

In the molecular analysis, *BRAF* mutations were exclusive to the PTC group (38.1%), and the *NRAS* mutations were present in both groups, although more frequent in the FTC group (28.6% *vs.* 9.5%).

Table 8 *- Comparison between the PTC and FTC, in relation to the variables age, tumour size, tumour capsule, tumour capsule invasion, lymph node metastasis, extra-thyroid invasion and BRAF and NRAS mutational status.*

Characteristics		Tumour diagnosis		p-value
		PTC	FTC	
Age	**Mean (years)**	**39.2**	**51.7**	**0.011**
Tumour size	**Mean (cm)**	**2.7**	**3.7**	**0.017**
Tumour capsule	**Absent**	**40** 64.5%	**0** 0.0%	**0.000**
	Present	**22** 35.5%	**14** 100.0%	
Tumour capsule invasion	**Absent**	**5** 33.3%	**0** 0.0%	**0.025**
	Present	**10** 66.7%	**14** 100.0%	
Lymph node metastasis	**Absent**	**24** 55.8%	**12** 100.0%	**0.003**
	Present	**19** 44.2%	**0** 0.0%	
Extra-thyroid Invasion	**Absent**	**33** 63.5%	**14** 100.0%	**0.004**
	Present	**19** 36.5%	**0** 0.0%	
BRAF	**WT**	**39** 61.9%	**14** 100.0%	**0.003**
	Mutated	**24** 38.1%	**0** 0.0%	
NRAS	**WT**	**57** 90.5%	**10** 71.4%	**0.077**
	Mutated	**6** 9.5%	**4** 28.6%	

Legend: Statistical differences are considered as significant when p-value <0.05; PTC, papillary thyroid carcinoma; FTC, follicular thyroid carcinoma; WT, wild type.

The statistical analysis of the clinico-pathological features and mutational status of the tumours was also performed considering the PTC, FVPTC and FTC groups individually. The PTCs had the highest number of statistical relationships, as this was the group with the higher number of tumour samples, when compared to the FVPTC and FTC groups although some associations have no immediate biological significance as previously mentioned (please see Supplementary Tables 16-20).

Table 9; 10; 11; 16; 17 and 18 present the significant relations found in the PTC group, which will be further addressed.

When the relation between gender and the other variables was analysed (Table 9) it was possible to identify several statistically significant differences between the male and

female gender. The male patients were younger (28 years *vs.* 41 years) and had larger tumours (4.5cm *vs.* 2.4cm) than the female patients. Tumours from male patients also harboured more frequently vascular invasion (100.0% *vs.* 49.1%) than the tumours from female patients.

In the *BRAF* genetic analysis, none of the tumours from male patients harboured *BRAF* mutations, while 41.8% of the tumours from female patients harboured these mutations.

Table 9 *- Comparison between male and female patients with PTC, in relation to the variables age, tumour size, vascular invasion and BRAF mutational status.*

Characteristics		**Gender**		**p-value**
		Male	**Female**	
Age	**Mean (years)**	**28.0**	**40.6**	**0.045**
Tumour size	**Mean (cm)**	**4.5**	**2.4**	**0.003**
Vascular invasion	**Absent**	**0** 0.0%	**28** 50.9%	**0.020**
	Present	**6** 100.%	**27** 49.1%	
BRAF	**WT**	**7** 100.0%	**32** 58.2%	**0.031**
	Mutated	**0** 0.0%	**23** 41.8%	

Legend: Statistical differences are considered as significant when p-value <0.05; WT, wild type.

When variable lymph node metastasis was compared with the others (Table 10), it was possible to observe that patients that had lymph node metastasis were younger (32 years *vs.* 44), harboured more frequently vascular invasion (78.9% *vs.* 39.1%), and more extra-thyroid invasion (44.4% *vs.* 7.1%) than the ones that did not harbour lymph node metastases.

For patients who harboured lymph node metastases only 21.1% of them were *BRAF* mutated, however all patients with positive tumours for the *RET/PTC* rearrangement harboured lymph node metastases, representing 38.6% of all patients with lymph node metastases.

In the analysis of the variable vascular invasion (Table 11), it was possible to take notice that patients with vascular invasion harboured larger tumours (2.1cm *vs.* 3.3cm); had more extra-thyroid invasion (57.1% *vs.* 13.0%) than the patients without vascular invasion; and also harboured less *BRAF* mutations (27.3% *vs* 51.7%) than patients with wild-type tumours.

Table 10 *- Comparison between PTC without or with lymph node metastasis, in relation to the variables age, vascular invasion, extra-thyroid invasion, BRAF and RET/PTC mutational status.*

Characteristics		Lymph Node Metastasis		p-value
		Absent	Present	
Age	**Mean (years)**	**44.2**	**32.2**	**0.017**
Vascular invasion	**Absent**	**14** 60.9%	**4** 21.1%	**0.010**
	Present	**9** 39.1%	**15** 78.9%	
Extra-thyroid invasion	**Absent**	**13** 92.9%	**10** 55.6%	**0.024**
	Present	**1** 7.1%	**8** 44.4%	
Multinodular goiter	**Absent**	**24** 100.0%	**13** 72.2%	**0.010**
	Present	**0** 0.0%	**5** 27.8%	
BRAF	**WT**	**11** 45.8%	**15** 78.9%	**0.028**
	Mutated	**13** 54.2%	**4** 21.1%	
RET/PTC1	**Negative**	**23** 100.0%	**12** 63.2%	**0.002**
	Positive	**0** 0.0%	**7** 36.8%	

Legend: Statistical differences are considered as significant when p-value <0.05; WT, wild type.

Table 11 *- Comparison between PTC without or with vascular invasion in relation to the variables tumour size, extra-thyroid invasion and BRAF mutational status.*

Characteristics		Vascular invasion		p-value
		Absent	Present	
Tumour size	**Mean (cm)**	**2.1**	**3.3**	**0,007**
Extra-thyroid invasion	**Absent**	**20** 87.0%	**12** 42.9%	**0,001**
	Present	**3** 13.0%	**16** 57.1%	
BRAF	**WT**	**14** 48.3%	**24** 72.7%	**0.043**
	Mutated	**15** 51.7%	**9** 27.3%	

Legend: Statistical differences are considered as significant when p-value <0.05; WT, wild type.

Tables 12; 19 and 20 present the relations found between the clinico-pathological features and mutational status found within the FVPTC group.

When the relation between gender and other variables was analysed (Table 12), was observed with statistical significance that male patients harboured more frequently vascular invasion than the female patients (75.0% vs 4.8%).

Table 12 - *Comparison between male and female patients with FVPTC, in relation to the variable vascular invasion.*

Characteristics		Gender		p-value
		Male	Female	
Vascular invasion	Absent	**1** 25.0%	**20** 95.2%	**0.007**
	Present	**3** 75.0%	**1** 4.8%	

Statistical differences are considered as significant when p-value <0.05

Table 13 presents the only significant relation found within the FTC group, when the features and mutational status were analysed.

When the number of tumours was analysed, a relation with *NRAS* mutational status was found. Patients with multiple tumours at diagnosis harboured more *NRAS* mutations (57.1% *vs*. 0.0%) than patients with single tumours.

Table 13 - *Comparison between single and multiple FTC in relation with the variable NRAS mutational status.*

Characteristics		Number of tumours		p-value
		Single	Multiple	
NRAS	WT	**7** 100.0%	**3** 42.9%	**0.035**
	Mutated	**0** 0.0%	**4** 57.1%	

Legend: Statistical differences are considered as significant when p-value <0.05; WT, wild-type.

When the analysis for *TERT* promoter mutations was performed, no significant relation was found, but a tendency with older age at diagnosis was observed. In this series there were few lesions that harboured these genetic alterations and for this analysis all tumours that harboured *TERT* promoter mutations that created a consensus binding site (*TERT* -124 C>T and -146 C>T) were considered. The FTA, FVPTC, metastasis of FVPTC and FTC cases were then considered in a parallel statistical analysis.

A significant association was found between *TERT* promoter mutations and patient's age (Table 21); patients that harboured these mutations were significantly older (71 years vs. 44 years old) than the patients that did no harbour these mutations.

Discussion

The main goal, to build a repository of biological material of thyroid tumours, and a database that harbours samples of thyroid tumours with clinico-pathological features and characterization of the genetic alterations, was accomplished with success. The repository has samples of DNA, RNA and proteins of 24 samples of benign tumours, diagnosed as FTA, 117 samples of malignant tumours and 30 samples of normal thyroid tissue.

The established standard protocols for the extraction of the nucleic acids revealed a success rate above 97.0%, which allowed the use of the extracted components in molecular studies subsequently conducted, and a confidence in the obtained results.

The construction of this database met the purpose to provide a platform of samples with clinico-pathological features and genetic characterization that will allow the selection of specific cases, with specific features or genetic alterations, in order to perform additional studies in thyroid tumours etiopathogenesis and progression.

The additional aim, characterization of the genetic alterations in thyroid tumours, namely the point mutations in *BRAF*, *NRAS* and *TERT* genes, achieved through the molecular analysis of the DNA samples had a rate of success of 100.0%, considering all the samples in which DNA extraction was possible (99.4%). The research of rearrangements was also effective in all the samples where RNA extraction was possible (97.7%) and the results were reliable, due to the verification of the quality of the cDNA, the positive control used in each reaction and the sequencing of the RT-PCR product. Thus, only 1 malignant tumour was excluded from the point mutations and rearrangements analysis, and 2 tumours were excluded from the rearrangements analysis.

Regarding the genetic analysis performed in this work, the frequency and genetic alterations found were, in the majority of the cases, in agreement with literature reports.

It was possible to report in the present work a frequency of 38.1% of *BRAF* (p.V600E) mutations in PTCs, and 4.0% in FVPTCs which meets the reports in the literature, being the most common alteration found the *BRAF* (p.V600E) (56); whereas the mutations on this gene are less frequent in FVPTC, less than 10% (57). It is also possible to report the frequency of 4.0% of *BRAF* (p.K601E) in FVPTC, which is described in the literature as a rare mutation, representing 1-2% in all PTCs (54).

In the current study the analysis of point mutations in codon 61 of the *NRAS* gene revealed frequencies of 4.2% in FTAs; 28.6% in FTCs and 24.0% in FVPTC, which are comparable, to the ones previously described in the literature (144). It was also possible to report a frequency of *NRAS* mutations of 33.3% in PDTCs and 33.3% in distant metastasis. Regardless the limited size of the series, these results may be related with previous studies that show a higher frequency of *RAS* mutations in advanced stages of the disease, since

the authors believe that such mutations leads to tumour progression, rather than tumour initiation (86-89).

The incidence found of *NRAS* mutations in the PTC group was 9.5%. It is stated in the literature that the *RAS* mutations are a rare molecular alteration in PTC, with an overall frequency of 10-20%, particularly in FVPTCs (92), and that papillary carcinomas harbouring *RAS* mutations almost always have a follicular variant histology (113). In the near future we intend to do the histological revision of all the slides available for these tumours, in order to clarify our results.

One out of 3 (33.3%) MTC cases analysed showed *NRAS* mutation. *RAS* mutations have been recently described in a percentage of *RET*-negative sporadic MTC, where these molecular alterations seem to be mutually exclusive (180). The *RAS* mutations described in MTC, were *KRAS* and *HRAS* point mutations (181), nevertheless, recently it was reported a 1.8% frequency of *NRAS* mutations in MTC that were concomitant with PTCs (182). We do not have information if the MTC from our series positive for *NRAS* mutation also presented a concomitant PTC and further work will be necessary to explore a putative association between concomitant PTC/MTC cases and *NRAS* mutations.

The frequency of mutations in the promoter region of *TERT* in our series (7.1% in FTC, 4.0% in FVPTC and 33.3% in distant metastases) was lower than in other series of DTCs previously reported (163, 170, 171, 173). The *TERT* -124C>T was the most common mutation, as previously described by our group (163). Contrary to other reports (163, 170, 171, 173), a *TERT* -124C>T mutation was found in a benign lesion, a FTA, that did not harbour any other genetic alteration. Similarly to our findings on this work, it was recently reported in the literature a frequency of *TERT* promoter mutations of 2% in FTAs and 17% in atypical FTAs (174). We think this is a very interesting finding that deserves further work in the future in order to ascertain the frequency of *TERT* promoter mutations in thyroid benign lesions and to verify if these mutations correlated with the age of the patients or with other particular clinico-pathological features.

As described in the literature the *RET/PTC* rearrangements prevalence can range from 13-46% in PTCs (56), where the most frequent rearrangement is the *RET/PTC1* (105). In this work *RET/PTC1* showed an incidence of 9.1% when compared with the incidence of 1.5% of *RET/PTC3*. *RET/PTC1* rearrangements were present in PTCs (14.5%) and in FVPTCs (4.2%) whereas *RET/PTC3* rearrangements were found in a PTC (1.6%) and in FVPTC (4.2%).

The *PAX8-PPARɣ* rearrangements were only found in tumours with follicular architecture, with an incidence of 4.2% in the FVPTCs and 7.1% in the FTCs, frequencies that are lower than the ones described in the literature (144, 145).

Some of our results in the characterization of the genetic alterations were interesting and open for discussion, such as the overlapping of genetic alterations and tumour heterogeneity.

One case of cPTC harboured the overlapping of a *BRAF* (p.V600E) with a polymorphism in the *TERT* promoter. The *TERT* -150 C>T polymorphism does not create a *de novo* binding site, however it has been described that some polymorphisms may predispose to a worse prognosis in bladder cancer (183), and it would be interesting to perform further studies of the presence of *TERT* promoter polymorphisms present in thyroid tumours and its possible effects.

In a FVPTC metastasis both *TERT* -146 C>T and *NRAS* (p.Q61R) mutations were found, unfortunately the sample of the primary tumour was not available for genetic characterization. Landa *et al.* found an association between *TERT* promoter and *NRAS* mutations in PDTC and UTC and Melo *et al.* reported an association between *TERT* promoter mutations and higher stages of the disease (173). It will be interesting in this case to have access to the primary tumour in order to see if it also harboured these two molecular alterations and how these alterations contributed to the progression of the disease.

Another interesting case to report was one cPTC that harboured the *BRAF* (p.V600E) and a *RET/PTC1*, similar results were described in a recent study that suggests that these dual mutations are not rare events in well- differentiated PTC, occurring in a frequency of 19.3% (184). In our work, this was not confirmed, since only one of the positive cases for *RET/PTC* rearrangement harboured the *BRAF* (p.V600E) mutation. It will be also interesting to verify if both genetic alterations are present in the same cell or if it reflects tumour heterogeneity.

Interestingly the only FTC positive for *PAX8-PPARɣ* rearrangement was also positive for *RET/PTC1* rearrangement. *RET/PTC* rearrangement distribution within the tumour may be quite heterogeneous, varying from involving almost all neoplastic cells, clonal *RET/PTC*, or being detected in only a small fraction of tumour cells, non-clonal *RET/PTC*, as advanced by Unger *et al. (185)* in post-Chernobyl cases and Zhu*, et al.* in sporadic cases (100). This heterogeneous pattern of *RET/PTC* rearrangement was found in previous studies in adenomas and in other benign thyroid lesions (100). It is also possible that the presence of those rearrangements reflect the morphologic differentiation of the tumours with some areas of the tumour with solid architecture harbouring the *RET/PTC1* rearrangement (132), and areas with follicular differentiation harbouring *PAX8-PPARɣ* enabling the identification of both rearrangements in the same tumour.

Still related to tumour heterogeneity, it was possible to identify in the case of a patient with two independent tumours, both with cPTC diagnosis, that only one of them harboured *BRAF* (p.V600E) mutation. In another patient with two independent tumours, both also with

cPTC diagnosis, both harboured a *BRAF* (p.V600E) mutation. These findings can be related with the often multicentric presentation of cPTC, that can be clonally independent from each other (186), and result in the presence of several lesions with similar or different molecular profiles.

For the statistical analysis we decided to consider only the three groups of tumours with the highest number of samples, the PTC (which included cPTC, diffuse sclerosing PTC, tall cell PTC, SVPTC and oncocytic PTC), the FVPTC and the FTC (which included FTC and oncocytic FTC).

Not all results, presented above will be discussed, as it would be necessary to increase the number of certain types of tumours, especially in the FVPTC and FTC groups, to report accurate and reliable data.

When the comparison between PTCs and FVPTCs was performed, it was possible to observe that patients with PTC had more often lymph node metastasis than the FVPTCs. As described in the literature, PTCs have a tendency to spread into lymphatic channels and in this way to lymph nodes, and this pattern of metastisation is found at a significant proportion of the cases at diagnosis (15). The FVPTCs can present two different growth patterns, infiltrative and encapsulated (17); the first tends to give rise to lymph node metastasis, and present a similar patterns with the PTCs; whereas the second tends to give rise, whenever displaying angioinvasiveness, to lung and bone metastasis, similar to the behaviour of FTCs (17).

Due to the high frequency of patients with PTCs that harboured lymph node metastasis, it was also expected an association with vascular invasion, which may include venous and/or lymphatic invasion, and extra-thyroid invasion as they are more invasive tumours. This relation was found when the PTC group was compared with the FVPTC and with the FTC group.

The differences found in the comparisons of the mutational status of the *BRAF* and *NRAS* in PTC *vs* FVPTC and PTC *vs* FTC were expected and in accordance with the published studies, *i.e*., mutations in the *BRAF* gene are common in PTCs and rare in the FVPTCs (57), and mutations on the *RAS* gene are rare in PTCs, more frequent in FVPTCs (92), and common genetic alterations in FTCs (83).

The FTCs were encapsulated and all of them harboured tumour capsule invasion, which was significantly different from PTCs. Nevertheless this association was not surprising, since the encapsulation of the tumour and tumour capsule invasion are characteristic criteria for the diagnosis of FTC (27). Although there were significant differences in the comparison between PTC, FVPTC and FTC, it is important to refer that the number of samples in the groups is not considerable, especially in the FVPTC and FTC group.

When the statistical analysis was performed within the PTC group, it was possible to verify that the male patients, although younger, harboured larger tumour and higher vascular invasion than the female patients. Although some studies have shown associations between the male gender and worse prognosis (187), this was not verified in other studies and in the present work, even though some associations are significant, the number of patients is not considerable to draw conclusions.

The patients with PTCs with *BRAF* mutations harboured less lymph node metastases and vascular invasion than patients with these alterations. These are interesting associations to explore further, due to the active discussion in the field about the clinico-pathologic associations of the *BRAF* mutations in PTC (64).

In the present work all *RET/PTC* positive tumours harboured lymph node metastasis. Some previous studies report the presence of *RET/PTC* rearrangement in younger patients and with higher rates of lymph node metastases (113), and given the present results it would be interesting to further study this possible association in sporadic PTCs(187).

TERT promoter mutations were significantly associated with older age of the patients, when all the 4 tumour samples with these mutations were considered. Although based in a small number of cases this finding is in accordance with what was previously described by our group, when Melo *et al.* reported an association between *TERT* promoter mutations and older age at diagnosis, besides other features associated with worse prognosis (larger tumours, higher frequency of distant metastases and higher tumour stage) (173).

Although the construction of a repository of biological material and database of thyroid tumours was successful, some difficulties and limitations were encountered during the course of the work. One limitation was the absence of histological review of the cases. This fact rise some uncertainty in the classifications since, as already mentioned, some diagnosis were done some decades ago, when the diagnostic criteria were different from those used nowadays. A future work to be done will be the histological review of all the cases what will, eventually, answer some of the questions previously raised.

Another challenge in this work was the successful extraction of the nucleic acids, due to the very long time of storage at -80°C for some samples (more than 20 years). Although it is reported that the storage at ultra-low temperatures may preserve high molecular weight nucleic acids and proteins, it is known that RNA may be more prone to degradation in these conditions (188). Furthermore, in several specimens, it is unknown the time that elapsed between the collection of the sample from the surgical room and the frozen storage, which may have been delayed for unknown reasons (189). One of the future perspectives is the extraction of nucleic acids and proteins of the oldest cases that remain

in the repository in order to, at least, prevent further degradation that can occur by long time storage at low temperatures.

Another difficulty encountered in the work was the insufficient information about the localization of the frozen fragment in relation to the whole tumour sample, *i.e*., most reports did not refer to the precise localization of the fragment selected for storage at -80°C. Although the general rule indicates that the sample was taken from the more relevant nodule this fact implies some uncertainty about the actual sampling of the tumour. However in the future we can validate the genetic alterations found in each frozen case by using the corresponding paraffin embedded material and correlate this information with that from the histological slides including the evaluation of the architectural pattern and genetic alterations found.

Conclusions

The organization of a repository of biological material of thyroid tumours was successful and the protocols of extraction of nucleic acids and proteins were established. These facts fulfilled the aim of the conception of a standard protocol that insured the quality and availability of diverse biological material (DNA, RNA and proteins) from the same tumour area to be applied in several projects developed by our group in this area.

Through the creation of the database, that includes the clinico-pathological information for each case and the information about the genetic alterations frequent in thyroid tumours, it was possible to analyse differences and associations between the collected data. In several aspects it was possible to verify concordance with the information already reported in the literature, whereas in others, our results brought the attention to several questions that need to be addressed further to get a full understanding of the mechanisms of initiation and progression of thyroid tumours. This could be achieved in the future with a larger repository and a more complete database, where the differences and associations could be proved, and where the samples could be applied in parallel projects to provide new insights on the evolution of these tumours.

In conclusion, the organization of a repository of biological material of thyroid tumours and the elaboration of a database was an achieved purpose with the establishment of successful standard protocols; nevertheless this is an ongoing work, where more cases must be added, the clinico-pathological characterization needs to be completed and the quick screening of the most common genetic alterations known in thyroid tumours must be fully performed.

Bibliographic references

1. Hegedus L. Clinical practice. The thyroid nodule. The New England journal of medicine. 2004;351(17):1764-71.
2. Negri E, Dal Maso L, Ron E, La Vecchia C, Mark SD, Preston-Martin S, et al. A pooled analysis of case-control studies of thyroid cancer. II. Menstrual and reproductive factors. Cancer causes & control : CCC. 1999;10(2):143-55.
3. Cancer of The Thyroid-SEER Stat Fact Sheets [cited 2014 June]. Available from: http://seer.cancer.gov/statfacts/html/thyro.html.
4. (RORENO). RORdN. Taxas de incidência de cancro na região Norte de Portugal no sexo femino (2008). [cited 2014]. Available from: http://www.roreno.com.pt/pt/estatisticas/graficos/top-10.html.
5. Tuttle RM, Ball DW, Byrd D, Dilawari RA, Doherty GM, Duh QY, et al. Thyroid carcinoma. Journal of the National Comprehensive Cancer Network : JNCCN. 2010;8(11):1228-74.
6. Tuttle RM, Tala H, Shah J, Leboeuf R, Ghossein R, Gonen M, et al. Estimating risk of recurrence in differentiated thyroid cancer after total thyroidectomy and radioactive iodine remnant ablation: using response to therapy variables to modify the initial risk estimates predicted by the new American Thyroid Association staging system. Thyroid : official journal of the American Thyroid Association. 2010;20(12):1341-9.
7. Morente MM, Mager R, Alonso S, Pezzella F, Spatz A, Knox K, et al. TuBaFrost 2: Standardising tissue collection and quality control procedures for a European virtual frozen tissue bank network. European journal of cancer. 2006;42(16):2684-91.
8. Vaught J, Rogers J, Carolin T, Compton C. Biobankonomics: developing a sustainable business model approach for the formation of a human tissue biobank. Journal of the National Cancer Institute Monographs. 2011;2011(42):24-31.
9. Hamburg MA, Collins FS. The path to personalized medicine. The New England journal of medicine. 2010;363(4):301-4.
10. DeLellis RA, Lloyd RV, Heitz PU, Eng C. World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of Endocrine Organs. Lyon: IARC Press; 2004.
11. Chan JKC, Hirokawa M, Evans H, Williams ED, Osamura Y, Cady B, et al. Follicular adenoma. In: DeLellis RA, Lloyd RV, Heitz PU, Eng C, editors. World Health ORganization Cassification of Tumours Pathology and Genetics Tumours of Endocrine Organs. Lyon, France2004.
12. Cheung CC, Ezzat S, Ramyar L, Freeman JL, Asa SL. Molecular basis off hurthle cell papillary thyroid carcinoma. The Journal of clinical endocrinology and metabolism. 2000;85(2):878-82.
13. Katoh R, Harach HR, Williams ED. Solitary, multiple, and familial oxyphil tumours of the thyroid gland. The Journal of pathology. 1998;186(3):292-9.
14. Castro P, Sansonetty F, Soares P, Dias A, Sobrinho-Simoes M. Fetal adenomas and minimally invasive follicular carcinomas of the thyroid frequently display a triploid or near triploid DNA pattern. Virchows Archiv : an international journal of pathology. 2001;438(4):336-42.
15. LiVolsi VA, Albores-Saavedra J, Asa SL, Baloch ZW, Sobrinho-Simões M, Wenig B, et al. Papillary carcinoma. In: DeLellis RA, Lloyd RV, Heitz PU, Eng C, editors. World Health Organization Classification of Tumours Pathology and Genetics Tumours of Endocrine Organs. IBSN 9 28322 416 7. Lyon, France: IARC PRESS; 2004. p. 54-66.
16. Baloch ZW, LiVolsi VA. Pathologic diagnosis of papillary thyroid carcinoma: today and tomorrow. Expert review of molecular diagnostics. 2005;5(4):573-84.
17. Liu J, Singh B, Tallini G, Carlson DL, Katabi N, Shaha A, et al. Follicular variant of papillary thyroid carcinoma: a clinicopathologic study of a problematic entity. Cancer. 2006;107(6):1255-64.
18. Ostrowski ML, Merino MJ. Tall cell variant of papillary thyroid carcinoma: a reassessment and immunohistochemical study with comparison to the usual type of

papillary carcinoma of the thyroid. The American journal of surgical pathology. 1996;20(8):964-74.
19. Furmanchuk AW, Averkin JI, Egloff B, Ruchti C, Abelin T, Schappi W, et al. Pathomorphological findings in thyroid cancers of children from the Republic of Belarus: a study of 86 cases occurring between 1986 ('post-Chernobyl') and 1991. Histopathology. 1992;21(5):401-8.
20. Berho M, Suster S. The oncocytic variant of papillary carcinoma of the thyroid: a clinicopathologic study of 15 cases. Human pathology. 1997;28(1):47-53.
21. Sobrinho-Simoes MA, Nesland JM, Holm R, Sambade MC, Johannessen JV. Hurthle cell and mitochondrion-rich papillary carcinomas of the thyroid gland: an ultrastructural and immunocytochemical study. Ultrastructural pathology. 1985;8(2-3):131-42.
22. de Biase D, Visani M, Pession A, Tallini G. Molecular diagnosis of carcinomas of the thyroid gland. Frontiers in bioscience. 2014;6:1-14.
23. Vu-Phan D, Grachtchouk V, Yu J, Colby LA, Wicha MS, Koenig RJ. The thyroid cancer PAX8-PPARG fusion protein activates Wnt/TCF-responsive cells that have a transformed phenotype. Endocrine-related cancer. 2013;20(5):725-39.
24. Dean DS, Hay ID. Prognostic indicators in differentiated thyroid carcinoma. Cancer control : journal of the Moffitt Cancer Center. 2000;7(3):229-39.
25. Hundahl SA, Fleming ID, Fremgen AM, Menck HR. A National Cancer Data Base report on 53,856 cases of thyroid carcinoma treated in the U.S., 1985-1995 [see commetns]. Cancer. 1998;83(12):2638-48.
26. Xing M. Molecular pathogenesis and mechanisms of thyroid cancer. Nature reviews Cancer. 2013;13(3):184-99.
27. Sobrinho-Simoes M, Asa SL, Kroll TG, Nikiforov Y, DeLellis RA, Farid P, et al. Follicular carcinoma. In: DeLellis RA, Lloyd RV, Heitz PU, Eng C, editors. World Health Organization Classification of Tumours Pathology and Genetics Tumours of Endocrine Organs. IBSN 9 28322 416 7. Lyon, France: IARC PRESS; 2004. p. 67-72.
28. Sobrinho Simões MA-S, J; Tallini, G; Santoro, M; Volante, M; Pilotti, S; Carcangiu, ML; Papotti, M; Matias-Guiu, X; Guiter, GE; Zakowski, M; Sakamoto, A. Poorly differentiated carcinoma. WHO Classification of Tumours Pathology and Genetics Tumours of Endocrine Organs. Lyon, France: IARC Press; 2004. p. 73-6.
29. Volante M, Collini P, Nikiforov YE, Sakamoto A, Kakudo K, Katoh R, et al. Poorly differentiated thyroid carcinoma: the Turin proposal for the use of uniform diagnostic criteria and an algorithmic diagnostic approach. The American journal of surgical pathology. 2007;31(8):1256-64.
30. Nagaiah G, Hossain A, Mooney CJ, Parmentier J, Remick SC. Anaplastic thyroid cancer: a review of epidemiology, pathogenesis, and treatment. Journal of oncology. 2011;2011:542358.
31. Sobrinho-Simoes M, Albores-Saavedra J, Tallini G, Santoro M, Volante M, Pilotti S, et al. Poorly differentiated carcinoma. In: DeLellis RA, Lloyd RV, Heitz PU, Eng C, editors. World Health Organization Classification of Tumours Pathology and Genetics Tumours of Endocrine Organs. IBSN 9 28322 416 7. Lyon, France: IARC PRESS; 2004. p. 73-6.
32. Costa AM, Herrero A, Fresno MF, Heymann J, Alvarez JA, Cameselle-Teijeiro J, et al. BRAF mutation associated with other genetic events identifies a subset of aggressive papillary thyroid carcinoma. Clinical endocrinology. 2008;68(4):618-34.
33. Sobrinho-Simoes M, Maximo V, Rocha AS, Trovisco V, Castro P, Preto A, et al. Intragenic mutations in thyroid cancer. Endocrinology and metabolism clinics of North America. 2008;37(2):333-62, viii.
34. Albores-Saavedra J, Gorraez de la Mora T, de la Torre-Rendon F, Gould E. Mixed medullary-papillary carcinoma of the thyroid: a previously unrecognized variant of thyroid carcinoma. Human pathology. 1990;21(11):1151-5.
35. Papotti M, Sambataro D, Pecchioni C, Bussolati G. The Pathology of Medullary Carcinoma of the Thyroid: Review of the Literature and Personal Experience on 62 Cases. Endocrine pathology. 1996;7(1):1-20.

36. Uribe M, Fenoglio-Preiser CM, Grimes M, Feind C. Medullary carcinoma of the thyroid gland. Clinical, pathological, and immunohistochemical features with review of the literature. The American journal of surgical pathology. 1985;9(8):577-94.
37. Bergholm U, Adami HO, Bergstrom R, Johansson H, Lundell G, Telenius-Berg M, et al. Clinical characteristics in sporadic and familial medullary thyroid carcinoma. A nationwide study of 249 patients in Sweden from 1959 through 1981. Cancer. 1989;63(6):1196-204.
38. Sosonkina N, Starenki D, Park JI. The Role of STAT3 in Thyroid Cancer. Cancers. 2014;6(1):526-44.
39. Hou P, Liu D, Shan Y, Hu S, Studeman K, Condouris S, et al. Genetic alterations and their relationship in the phosphatidylinositol 3-kinase/Akt pathway in thyroid cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2007;13(4):1161-70.
40. Knauf JA, Fagin JA. Role of MAPK pathway oncoproteins in thyroid cancer pathogenesis and as drug targets. Current opinion in cell biology. 2009;21(2):296-303.
41. Kimura ET, Nikiforova MN, Zhu Z, Knauf JA, Nikiforov YE, Fagin JA. High prevalence of BRAF mutations in thyroid cancer: genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signaling pathway in papillary thyroid carcinoma. Cancer Res. 2003;63(7):1454-7.
42. Murugan AK, Dong J, Xie J, Xing M. Uncommon GNAQ, MMP8, AKT3, EGFR, and PIK3R1 mutations in thyroid cancers. Endocrine pathology. 2011;22(2):97-102.
43. Soares P, Trovisco V, Rocha AS, Lima J, Castro P, Preto A, et al. BRAF mutations and RET/PTC rearrangements are alternative events in the etiopathogenesis of PTC. Oncogene. 2003;22(29):4578-80.
44. Xing M. BRAF mutation in thyroid cancer. Endocrine-related cancer. 2005;12(2):245-62.
45. Soares P, Lima J, Preto A, Castro P, Vinagre J, Celestino R, et al. Genetic alterations in poorly differentiated and undifferentiated thyroid carcinomas. Current genomics. 2011;12(8):609-17.
46. Marais R, Light Y, Paterson HF, Mason CS, Marshall CJ. Differential regulation of Raf-1, A-Raf, and B-Raf by oncogenic ras and tyrosine kinases. The Journal of biological chemistry. 1997;272(7):4378-83.
47. Nikiforov YE. Molecular diagnostics of thyroid tumors. Archives of pathology & laboratory medicine. 2011;135(5):569-77.
48. Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, et al. Mutations of the BRAF gene in human cancer. Nature. 2002;417(6892):949-54.
49. Dhillon AS, Kolch W. Oncogenic B-Raf mutations: crystal clear at last. Cancer cell. 2004;5(4):303-4.
50. Cohen Y, Xing M, Mambo E, Guo Z, Wu G, Trink B, et al. BRAF mutation in papillary thyroid carcinoma. Journal of the National Cancer Institute. 2003;95(8):625-7.
51. Fukushima T, Suzuki S, Mashiko M, Ohtake T, Endo Y, Takebayashi Y, et al. BRAF mutations in papillary carcinomas of the thyroid. Oncogene. 2003;22(41):6455-7.
52. Cantwell-Dorris ER, O'Leary JJ, Sheils OM. BRAFV600E: implications for carcinogenesis and molecular therapy. Molecular cancer therapeutics. 2011;10(3):385-94.
53. Wan PT, Garnett MJ, Roe SM, Lee S, Niculescu-Duvaz D, Good VM, et al. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. Cell. 2004;116(6):855-67.
54. Chiosea S, Nikiforova M, Zuo H, Ogilvie J, Gandhi M, Seethala RR, et al. A novel complex BRAF mutation detected in a solid variant of papillary thyroid carcinoma. Endocrine pathology. 2009;20(2):122-6.
55. Ciampi R, Knauf JA, Kerler R, Gandhi M, Zhu Z, Nikiforova MN, et al. Oncogenic AKAP9-BRAF fusion is a novel mechanism of MAPK pathway activation in thyroid cancer. The Journal of clinical investigation. 2005;115(1):94-101.
56. Kondo T, Ezzat S, Asa SL. Pathogenetic mechanisms in thyroid follicular-cell neoplasia. Nature reviews Cancer. 2006;6(4):292-306.

57. Trovisco V, Vieira de Castro I, Soares P, Maximo V, Silva P, Magalhaes J, et al. BRAF mutations are associated with some histological types of papillary thyroid carcinoma. The Journal of pathology. 2004;202(2):247-51.
58. Penko K, Livezey J, Fenton C, Patel A, Nicholson D, Flora M, et al. BRAF mutations are uncommon in papillary thyroid cancer of young patients. Thyroid : official journal of the American Thyroid Association. 2005;15(4):320-5.
59. Nikiforova MN, Ciampi R, Salvatore G, Santoro M, Gandhi M, Knauf JA, et al. Low prevalence of BRAF mutations in radiation-induced thyroid tumors in contrast to sporadic papillary carcinomas. Cancer letters. 2004;209(1):1-6.
60. Knauf JA, Ma X, Smith EP, Zhang L, Mitsutake N, Liao XH, et al. Targeted expression of BRAFV600E in thyroid cells of transgenic mice results in papillary thyroid cancers that undergo dedifferentiation. Cancer Res. 2005;65(10):4238-45.
61. Mitsutake N, Knauf JA, Mitsutake S, Mesa C, Jr., Zhang L, Fagin JA. Conditional BRAFV600E expression induces DNA synthesis, apoptosis, dedifferentiation, and chromosomal instability in thyroid PCCL3 cells. Cancer Res. 2005;65(6):2465-73.
62. Liu D, Liu Z, Condouris S, Xing M. BRAF V600E maintains proliferation, transformation, and tumorigenicity of BRAF-mutant papillary thyroid cancer cells. The Journal of clinical endocrinology and metabolism. 2007;92(6):2264-71.
63. Charles RP, Iezza G, Amendola E, Dankort D, McMahon M. Mutationally activated BRAF(V600E) elicits papillary thyroid cancer in the adult mouse. Cancer Res. 2011;71(11):3863-71.
64. Handkiewicz-Junak D, Czarniecka A, Jarzab B. Molecular prognostic markers in papillary and follicular thyroid cancer: Current status and future directions. Molecular and cellular endocrinology. 2010;322(1-2):8-28.
65. Lupi C, Giannini R, Ugolini C, Proietti A, Berti P, Minuto M, et al. Association of BRAF V600E mutation with poor clinicopathological outcomes in 500 consecutive cases of papillary thyroid carcinoma. The Journal of clinical endocrinology and metabolism. 2007;92(11):4085-90.
66. Xing M. BRAF mutation in papillary thyroid cancer: pathogenic role, molecular bases, and clinical implications. Endocrine reviews. 2007;28(7):742-62.
67. Espinosa AV, Porchia L, Ringel MD. Targeting BRAF in thyroid cancer. British journal of cancer. 2007;96(1):16-20.
68. Mitsiades CS, Negri J, McMullan C, McMillin DW, Sozopoulos E, Fanourakis G, et al. Targeting BRAFV600E in thyroid carcinoma: therapeutic implications. Molecular cancer therapeutics. 2007;6(3):1070-8.
69. Ricarte-Filho JC, Ryder M, Chitale DA, Rivera M, Heguy A, Ladanyi M, et al. Mutational profile of advanced primary and metastatic radioactive iodine-refractory thyroid cancers reveals distinct pathogenetic roles for BRAF, PIK3CA, and AKT1. Cancer Res. 2009;69(11):4885-93.
70. Durante C, Puxeddu E, Ferretti E, Morisi R, Moretti S, Bruno R, et al. BRAF mutations in papillary thyroid carcinomas inhibit genes involved in iodine metabolism. The Journal of clinical endocrinology and metabolism. 2007;92(7):2840-3.
71. Riesco-Eizaguirre G, Gutierrez-Martinez P, Garcia-Cabezas MA, Nistal M, Santisteban P. The oncogene BRAF V600E is associated with a high risk of recurrence and less differentiated papillary thyroid carcinoma due to the impairment of Na+/I- targeting to the membrane. Endocrine-related cancer. 2006;13(1):257-69.
72. Kebebew E, Weng J, Bauer J, Ranvier G, Clark OH, Duh QY, et al. The prevalence and prognostic value of BRAF mutation in thyroid cancer. Annals of surgery. 2007;246(3):466-70; discussion 70-1.
73. Kim TY, Kim WB, Rhee YS, Song JY, Kim JM, Gong G, et al. The BRAF mutation is useful for prediction of clinical recurrence in low-risk patients with conventional papillary thyroid carcinoma. Clinical endocrinology. 2006;65(3):364-8.
74. Elisei R, Ugolini C, Viola D, Lupi C, Biagini A, Giannini R, et al. BRAF(V600E) mutation and outcome of patients with papillary thyroid carcinoma: a 15-year median follow-up study. The Journal of clinical endocrinology and metabolism. 2008;93(10):3943-9.

75. Nikiforova MN, Kimura ET, Gandhi M, Biddinger PW, Knauf JA, Basolo F, et al. BRAF mutations in thyroid tumors are restricted to papillary carcinomas and anaplastic or poorly differentiated carcinomas arising from papillary carcinomas. The Journal of clinical endocrinology and metabolism. 2003;88(11):5399-404.
76. Soares P, Trovisco V, Rocha AS, Feijao T, Rebocho AP, Fonseca E, et al. BRAF mutations typical of papillary thyroid carcinoma are more frequently detected in undifferentiated than in insular and insular-like poorly differentiated carcinomas. Virchows Archiv : an international journal of pathology. 2004;444(6):572-6.
77. Xing M. Identifying genetic alterations in poorly differentiated thyroid cancer: a rewarding pursuit. The Journal of clinical endocrinology and metabolism. 2009;94(12):4661-4.
78. Lemoine NR, Mayall ES, Wyllie FS, Farr CJ, Hughes D, Padua RA, et al. Activated ras oncogenes in human thyroid cancers. Cancer Res. 1988;48(16):4459-63.
79. Suarez HG, du Villard JA, Severino M, Caillou B, Schlumberger M, Tubiana M, et al. Presence of mutations in all three ras genes in human thyroid tumors. Oncogene. 1990;5(4):565-70.
80. Suarez HG, Du Villard JA, Caillou B, Schlumberger M, Tubiana M, Parmentier C, et al. Detection of activated ras oncogenes in human thyroid carcinomas. Oncogene. 1988;2(4):403-6.
81. Shi YF, Zou MJ, Schmidt H, Juhasz F, Stensky V, Robb D, et al. High rates of ras codon 61 mutation in thyroid tumors in an iodide-deficient area. Cancer Res. 1991;51(10):2690-3.
82. Suchy B, Waldmann V, Klugbauer S, Rabes HM. Absence of RAS and p53 mutations in thyroid carcinomas of children after Chernobyl in contrast to adult thyroid tumours. British journal of cancer. 1998;77(6):952-5.
83. Nikiforova MN, Nikiforov YE. Molecular diagnostics and predictors in thyroid cancer. Thyroid : official journal of the American Thyroid Association. 2009;19(12):1351-61.
84. Elsheikh TM, Asa SL, Chan JK, DeLellis RA, Heffess CS, LiVolsi VA, et al. Interobserver and intraobserver variation among experts in the diagnosis of thyroid follicular lesions with borderline nuclear features of papillary carcinoma. American journal of clinical pathology. 2008;130(5):736-44.
85. Lloyd RV, Erickson LA, Casey MB, Lam KY, Lohse CM, Asa SL, et al. Observer variation in the diagnosis of follicular variant of papillary thyroid carcinoma. The American journal of surgical pathology. 2004;28(10):1336-40.
86. Garcia-Rostan G, Zhao H, Camp RL, Pollan M, Herrero A, Pardo J, et al. ras mutations are associated with aggressive tumor phenotypes and poor prognosis in thyroid cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2003;21(17):3226-35.
87. Volante M, Rapa I, Gandhi M, Bussolati G, Giachino D, Papotti M, et al. RAS mutations are the predominant molecular alteration in poorly differentiated thyroid carcinomas and bear prognostic impact. The Journal of clinical endocrinology and metabolism. 2009;94(12):4735-41.
88. Abulaiti A, Fikaris AJ, Tsygankova OM, Meinkoth JL. Ras induces chromosome instability and abrogation of the DNA damage response. Cancer Res. 2006;66(21):10505-12.
89. Vitagliano D, Portella G, Troncone G, Francione A, Rossi C, Bruno A, et al. Thyroid targeting of the N-ras(Gln61Lys) oncogene in transgenic mice results in follicular tumors that progress to poorly differentiated carcinomas. Oncogene. 2006;25(39):5467-74.
90. Gupta N, Dasyam AK, Carty SE, Nikiforova MN, Ohori NP, Armstrong M, et al. RAS mutations in thyroid FNA specimens are highly predictive of predominantly low-risk follicular-pattern cancers. The Journal of clinical endocrinology and metabolism. 2013;98(5):E914-22.
91. Howitt BE, Jia Y, Sholl LM, Barletta JA. Molecular alterations in partially-encapsulated or well-circumscribed follicular variant of papillary thyroid carcinoma. Thyroid : official journal of the American Thyroid Association. 2013;23(10):1256-62.

92. Zhu Z, Gandhi M, Nikiforova MN, Fischer AH, Nikiforov YE. Molecular profile and clinical-pathologic features of the follicular variant of papillary thyroid carcinoma. An unusually high prevalence of ras mutations. American journal of clinical pathology. 2003;120(1):71-7.
93. Basolo F, Pisaturo F, Pollina LE, Fontanini G, Elisei R, Molinaro E, et al. N-ras mutation in poorly differentiated thyroid carcinomas: correlation with bone metastases and inverse correlation to thyroglobulin expression. Thyroid : official journal of the American Thyroid Association. 2000;10(1):19-23.
94. Manenti G, Pilotti S, Re FC, Della Porta G, Pierotti MA. Selective activation of ras oncogenes in follicular and undifferentiated thyroid carcinomas. European journal of cancer. 1994;30A(7):987-93.
95. Arighi E, Borrello MG, Sariola H. RET tyrosine kinase signaling in development and cancer. Cytokine & growth factor reviews. 2005;16(4-5):441-67.
96. Durbec P, Marcos-Gutierrez CV, Kilkenny C, Grigoriou M, Wartiowaara K, Suvanto P, et al. GDNF signalling through the Ret receptor tyrosine kinase. Nature. 1996;381(6585):789-93.
97. Trupp M, Arenas E, Fainzilber M, Nilsson AS, Sieber BA, Grigoriou M, et al. Functional receptor for GDNF encoded by the c-ret proto-oncogene. Nature. 1996;381(6585):785-9.
98. Fusco A, Grieco M, Santoro M, Berlingieri MT, Pilotti S, Pierotti MA, et al. A new oncogene in human thyroid papillary carcinomas and their lymph-nodal metastases. Nature. 1987;328(6126):170-2.
99. Marotta V, Guerra A, Sapio MR, Vitale M. RET/PTC rearrangement in benign and malignant thyroid diseases: a clinical standpoint. European journal of endocrinology / European Federation of Endocrine Societies. 2011;165(4):499-507.
100. Zhu Z, Ciampi R, Nikiforova MN, Gandhi M, Nikiforov YE. Prevalence of RET/PTC rearrangements in thyroid papillary carcinomas: effects of the detection methods and genetic heterogeneity. The Journal of clinical endocrinology and metabolism. 2006;91(9):3603-10.
101. Ciampi R, Giordano TJ, Wikenheiser-Brokamp K, Koenig RJ, Nikiforov YE. HOOK3-RET: a novel type of RET/PTC rearrangement in papillary thyroid carcinoma. Endocrine-related cancer. 2007;14(2):445-52.
102. Hamatani K, Eguchi H, Koyama K, Mukai M, Nakachi K, Kusunoki Y. A novel RET rearrangement (ACBD5/RET) by pericentric inversion, inv(10)(p12.1;q11.2), in papillary thyroid cancer from an atomic bomb survivor exposed to high-dose radiation. Oncology reports. 2014.
103. Basolo F, Giannini R, Monaco C, Melillo RM, Carlomagno F, Pancrazi M, et al. Potent mitogenicity of the RET/PTC3 oncogene correlates with its prevalence in tall-cell variant of papillary thyroid carcinoma. The American journal of pathology. 2002;160(1):247-54.
104. Jhiang SM, Sagartz JE, Tong Q, Parker-Thornburg J, Capen CC, Cho JY, et al. Targeted expression of the ret/PTC1 oncogene induces papillary thyroid carcinomas. Endocrinology. 1996;137(1):375-8.
105. Santoro M, Melillo RM, Fusco A. RET/PTC activation in papillary thyroid carcinoma: European Journal of Endocrinology Prize Lecture. European journal of endocrinology / European Federation of Endocrine Societies. 2006;155(5):645-53.
106. Bongarzone I, Vigneri P, Mariani L, Collini P, Pilotti S, Pierotti MA. RET/NTRK1 rearrangements in thyroid gland tumors of the papillary carcinoma family: correlation with clinicopathological features. Clinical cancer research : an official journal of the American Association for Cancer Research. 1998;4(1):223-8.
107. Tallini G, Santoro M, Helie M, Carlomagno F, Salvatore G, Chiappetta G, et al. RET/PTC oncogene activation defines a subset of papillary thyroid carcinomas lacking evidence of progression to poorly differentiated or undifferentiated tumor phenotypes. Clinical cancer research : an official journal of the American Association for Cancer Research. 1998;4(2):287-94.

108. Bunone G, Uggeri M, Mondellini P, Pierotti MA, Bongarzone I. RET receptor expression in thyroid follicular epithelial cell-derived tumors. Cancer Res. 2000;60(11):2845-9.
109. Grieco M, Santoro M, Berlingieri MT, Melillo RM, Donghi R, Bongarzone I, et al. PTC is a novel rearranged form of the ret proto-oncogene and is frequently detected in vivo in human thyroid papillary carcinomas. Cell. 1990;60(4):557-63.
110. Melillo RM, Castellone MD, Guarino V, De Falco V, Cirafici AM, Salvatore G, et al. The RET/PTC-RAS-BRAF linear signaling cascade mediates the motile and mitogenic phenotype of thyroid cancer cells. The Journal of clinical investigation. 2005;115(4):1068-81.
111. Miyagi E, Braga-Basaria M, Hardy E, Vasko V, Burman KD, Jhiang S, et al. Chronic expression of RET/PTC 3 enhances basal and insulin-stimulated PI3 kinase/AKT signaling and increases IRS-2 expression in FRTL-5 thyroid cells. Molecular carcinogenesis. 2004;41(2):98-107.
112. Ciampi R, Nikiforov YE. RET/PTC rearrangements and BRAF mutations in thyroid tumorigenesis. Endocrinology. 2007;148(3):936-41.
113. Adeniran AJ, Zhu Z, Gandhi M, Steward DL, Fidler JP, Giordano TJ, et al. Correlation between genetic alterations and microscopic features, clinical manifestations, and prognostic characteristics of thyroid papillary carcinomas. The American journal of surgical pathology. 2006;30(2):216-22.
114. Nikiforov YE, Rowland JM, Bove KE, Monforte-Munoz H, Fagin JA. Distinct pattern of ret oncogene rearrangements in morphological variants of radiation-induced and sporadic thyroid papillary carcinomas in children. Cancer Res. 1997;57(9):1690-4.
115. Hamatani K, Eguchi H, Ito R, Mukai M, Takahashi K, Taga M, et al. RET/PTC rearrangements preferentially occurred in papillary thyroid cancer among atomic bomb survivors exposed to high radiation dose. Cancer Res. 2008;68(17):7176-82.
116. Nikiforova MN, Stringer JR, Blough R, Medvedovic M, Fagin JA, Nikiforov YE. Proximity of chromosomal loci that participate in radiation-induced rearrangements in human cells. Science. 2000;290(5489):138-41.
117. Gandhi M, Medvedovic M, Stringer JR, Nikiforov YE. Interphase chromosome folding determines spatial proximity of genes participating in carcinogenic RET/PTC rearrangements. Oncogene. 2006;25(16):2360-6.
118. Thomas GA, Bunnell H, Cook HA, Williams ED, Nerovnya A, Cherstvoy ED, et al. High prevalence of RET/PTC rearrangements in Ukrainian and Belarussian post-Chernobyl thyroid papillary carcinomas: a strong correlation between RET/PTC3 and the solid-follicular variant. The Journal of clinical endocrinology and metabolism. 1999;84(11):4232-8.
119. Sugg SL, Ezzat S, Rosen IB, Freeman JL, Asa SL. Distinct multiple RET/PTC gene rearrangements in multifocal papillary thyroid neoplasia. The Journal of clinical endocrinology and metabolism. 1998;83(11):4116-22.
120. Sugg SL, Zheng L, Rosen IB, Freeman JL, Ezzat S, Asa SL. ret/PTC-1, -2, and -3 oncogene rearrangements in human thyroid carcinomas: implications for metastatic potential? The Journal of clinical endocrinology and metabolism. 1996;81(9):3360-5.
121. Fusco A, Chiappetta G, Hui P, Garcia-Rostan G, Golden L, Kinder BK, et al. Assessment of RET/PTC oncogene activation and clonality in thyroid nodules with incomplete morphological evidence of papillary carcinoma: a search for the early precursors of papillary cancer. The American journal of pathology. 2002;160(6):2157-67.
122. Fischer AH, Bond JA, Taysavang P, Battles OE, Wynford-Thomas D. Papillary thyroid carcinoma oncogene (RET/PTC) alters the nuclear envelope and chromatin structure. The American journal of pathology. 1998;153(5):1443-50.
123. Santoro M, Chiappetta G, Cerrato A, Salvatore D, Zhang L, Manzo G, et al. Development of thyroid papillary carcinomas secondary to tissue-specific expression of the RET/PTC1 oncogene in transgenic mice. Oncogene. 1996;12(8):1821-6.
124. Soares P, Fonseca E, Wynford-Thomas D, Sobrinho-Simoes M. Sporadic ret-rearranged papillary carcinoma of the thyroid: a subset of slow growing, less aggressive thyroid neoplasms? The Journal of pathology. 1998;185(1):71-8.

125. Santoro M, Sabino N, Ishizaka Y, Ushijima T, Carlomagno F, Cerrato A, et al. Involvement of RET oncogene in human tumours: specificity of RET activation to thyroid tumours. British journal of cancer. 1993;68(3):460-4.
126. Wang J, Knauf JA, Basu S, Puxeddu E, Kuroda H, Santoro M, et al. Conditional expression of RET/PTC induces a weak oncogenic drive in thyroid PCCL3 cells and inhibits thyrotropin action at multiple levels. Molecular endocrinology. 2003;17(7):1425-36.
127. Santoro M, Papotti M, Chiappetta G, Garcia-Rostan G, Volante M, Johnson C, et al. RET activation and clinicopathologic features in poorly differentiated thyroid tumors. The Journal of clinical endocrinology and metabolism. 2002;87(1):370-9.
128. Castellone MD, Santoro M. Dysregulated RET signaling in thyroid cancer. Endocrinology and metabolism clinics of North America. 2008;37(2):363-74, viii.
129. Richardson DS, Gujral TS, Peng S, Asa SL, Mulligan LM. Transcript level modulates the inherent oncogenicity of RET/PTC oncoproteins. Cancer Res. 2009;69(11):4861-9.
130. Chiappetta G, Toti P, Cetta F, Giuliano A, Pentimalli F, Amendola I, et al. The RET/PTC oncogene is frequently activated in oncocytic thyroid tumors (Hurthle cell adenomas and carcinomas), but not in oncocytic hyperplastic lesions. The Journal of clinical endocrinology and metabolism. 2002;87(1):364-9.
131. Musholt PB, Imkamp F, von Wasielewski R, Schmid KW, Musholt TJ. RET rearrangements in archival oxyphilic thyroid tumors: new insights in tumorigenesis and classification of Hurthle cell carcinomas? Surgery. 2003;134(6):881-9; discussion 9.
132. de Vries MM, Celestino R, Castro P, Eloy C, Maximo V, van der Wal JE, et al. RET/PTC rearrangement is prevalent in follicular Hurthle cell carcinomas. Histopathology. 2012;61(5):833-43.
133. Garcia-Rostan G, Sobrinho-Simões M. Poorly differentiated thyroid carcinoma: an evolving entity. Diagnostic Histopathology. 2010;17:114-12310.1016.
134. Mochizuki K, Kondo T, Nakazawa T, Iwashina M, Kawasaki T, Nakamura N, et al. RET rearrangements and BRAF mutation in undifferentiated thyroid carcinomas having papillary carcinoma components. Histopathology. 2010;57(3):444-50.
135. Di Palma T, Filippone MG, Pierantoni GM, Fusco A, Soddu S, Zannini M. Pax8 has a critical role in epithelial cell survival and proliferation. Cell death & disease. 2013;4:e729.
136. Au AY, McBride C, Wilhelm KG, Jr., Koenig RJ, Speller B, Cheung L, et al. PAX8-peroxisome proliferator-activated receptor gamma (PPARgamma) disrupts normal PAX8 or PPARgamma transcriptional function and stimulates follicular thyroid cell growth. Endocrinology. 2006;147(1):367-76.
137. Kroll TG, Sarraf P, Pecciarini L, Chen CJ, Mueller E, Spiegelman BM, et al. PAX8-PPARgamma1 fusion oncogene in human thyroid carcinoma [corrected]. Science. 2000;289(5483):1357-60.
138. Fagin JA, Mitsiades N. Molecular pathology of thyroid cancer: diagnostic and clinical implications. Best practice & research Clinical endocrinology & metabolism. 2008;22(6):955-69.
139. Lui WO, Zeng L, Rehrmann V, Deshpande S, Tretiakova M, Kaplan EL, et al. CREB3L2-PPARgamma fusion mutation identifies a thyroid signaling pathway regulated by intramembrane proteolysis. Cancer Res. 2008;68(17):7156-64.
140. French CA, Alexander EK, Cibas ES, Nose V, Laguette J, Faquin W, et al. Genetic and biological subgroups of low-stage follicular thyroid cancer. The American journal of pathology. 2003;162(4):1053-60.
141. Marques AR, Espadinha C, Frias MJ, Roque L, Catarino AL, Sobrinho LG, et al. Underexpression of peroxisome proliferator-activated receptor (PPAR)gamma in PAX8/PPARgamma-negative thyroid tumours. British journal of cancer. 2004;91(4):732-8.
142. Nikiforova MN, Lynch RA, Biddinger PW, Alexander EK, Dorn GW, 2nd, Tallini G, et al. RAS point mutations and PAX8-PPAR gamma rearrangement in thyroid tumors: evidence for distinct molecular pathways in thyroid follicular carcinoma. The Journal of clinical endocrinology and metabolism. 2003;88(5):2318-26.
143. Sahin M, Allard BL, Yates M, Powell JG, Wang XL, Hay ID, et al. PPARgamma staining as a surrogate for PAX8/PPARgamma fusion oncogene expression in follicular

neoplasms: clinicopathological correlation and histopathological diagnostic value. The Journal of clinical endocrinology and metabolism. 2005;90(1):463-8.
144. Castro P, Rebocho AP, Soares RJ, Magalhaes J, Roque L, Trovisco V, et al. PAX8-PPARgamma rearrangement is frequently detected in the follicular variant of papillary thyroid carcinoma. The Journal of clinical endocrinology and metabolism. 2006;91(1):213-20.
145. Eberhardt NL, Grebe SK, McIver B, Reddi HV. The role of the PAX8/PPARgamma fusion oncogene in the pathogenesis of follicular thyroid cancer. Molecular and cellular endocrinology. 2010;321(1):50-6.
146. Cassol CA, Guo M, Ezzat S, Asa SL. GNAq mutations are not identified in papillary thyroid carcinomas and hyperfunctioning thyroid nodules. Endocrine pathology. 2010;21(4):250-2.
147. Dumont JE, Maenhaut C, Pirson I, Baptist M, Roger PP. Growth factors controlling the thyroid gland. Bailliere's clinical endocrinology and metabolism. 1991;5(4):727-54.
148. Preto A, Cameselle-Teijeiro J, Moldes-Boullosa J, Soares P, Cameselle-Teijeiro JF, Silva P, et al. Telomerase expression and proliferative activity suggest a stem cell role for thyroid solid cell nests. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc. 2004;17(7):819-26.
149. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.
150. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, et al. Specific association of human telomerase activity with immortal cells and cancer. Science. 1994;266(5193):2011-5.
151. Shay JW, Bacchetti S. A survey of telomerase activity in human cancer. European journal of cancer. 1997;33(5):787-91.
152. Brousset P, Chaouche N, Leprat F, Branet-Brousset F, Trouette H, Zenou RC, et al. Telomerase activity in human thyroid carcinomas originating from the follicular cells. The Journal of clinical endocrinology and metabolism. 1997;82(12):4214-6.
153. Saji M, Xydas S, Westra WH, Liang CK, Clark DP, Udelsman R, et al. Human telomerase reverse transcriptase (hTERT) gene expression in thyroid neoplasms. Clinical cancer research : an official journal of the American Association for Cancer Research. 1999;5(6):1483-9.
154. Umbricht CB, Saji M, Westra WH, Udelsman R, Zeiger MA, Sukumar S. Telomerase activity: a marker to distinguish follicular thyroid adenoma from carcinoma. Cancer Res. 1997;57(11):2144-7.
155. Podlevsky JD, Chen JJ. It all comes together at the ends: telomerase structure, function, and biogenesis. Mutation research. 2012;730(1-2):3-11.
156. Masutomi K, Kaneko S, Hayashi N, Yamashita T, Shirota Y, Kobayashi K, et al. Telomerase activity reconstituted in vitro with purified human telomerase reverse transcriptase and human telomerase RNA component. The Journal of biological chemistry. 2000;275(29):22568-73.
157. Weinrich SL, Pruzan R, Ma L, Ouellette M, Tesmer VM, Holt SE, et al. Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTRT. Nature genetics. 1997;17(4):498-502.
158. Hahn WC, Meyerson M. Telomerase activation, cellular immortalization and cancer. Annals of medicine. 2001;33(2):123-9.
159. Zhu J, Zhao Y, Wang S. Chromatin and epigenetic regulation of the telomerase reverse transcriptase gene. Protein & cell. 2010;1(1):22-32.
160. Horn S, Figl A, Rachakonda PS, Fischer C, Sucker A, Gast A, et al. TERT promoter mutations in familial and sporadic melanoma. Science. 2013;339(6122):959-61.
161. Huang FW, Hodis E, Xu MJ, Kryukov GV, Chin L, Garraway LA. Highly recurrent TERT promoter mutations in human melanoma. Science. 2013;339(6122):957-9.
162. Killela PJ, Reitman ZJ, Jiao Y, Bettegowda C, Agrawal N, Diaz LA, Jr., et al. TERT promoter mutations occur frequently in gliomas and a subset of tumors derived from cells

with low rates of self-renewal. Proceedings of the National Academy of Sciences of the United States of America. 2013;110(15):6021-6.
163. Vinagre J, Almeida A, Populo H, Batista R, Lyra J, Pinto V, et al. Frequency of TERT promoter mutations in human cancers. Nature communications. 2013;4:2185.
164. Mocellin S, Pooley KA, Nitti D. Telomerase and the search for the end of cancer. Trends in molecular medicine. 2013;19(2):125-33.
165. Smekalova EM, Shubernetskaya OS, Zvereva MI, Gromenko EV, Rubtsova MP, Dontsova OA. Telomerase RNA biosynthesis and processing. Biochemistry Biokhimiia. 2012;77(10):1120-8.
166. Capezzone M, Cantara S, Marchisotta S, Busonero G, Formichi C, Benigni M, et al. Telomere length in neoplastic and nonneoplastic tissues of patients with familial and sporadic papillary thyroid cancer. The Journal of clinical endocrinology and metabolism. 2011;96(11):E1852-6.
167. Aogi K, Kitahara K, Urquidi V, Tarin D, Goodison S. Comparison of telomerase and CD44 expression as diagnostic tumor markers in lesions of the thyroid. Clinical cancer research : an official journal of the American Association for Cancer Research. 1999;5(10):2790-7.
168. Hoang-Vu C, Boltze C, Gimm O, Poremba C, Dockhorn-Dworniczak B, Kohrle J, et al. Expression of telomerase genes in thyroid carcinoma. International journal of oncology. 2002;21(2):265-72.
169. Takano T, Ito Y, Matsuzuka F, Miya A, Kobayashi K, Yoshida H, et al. Quantitative measurement of telomerase reverse transcriptase, thyroglobulin and thyroid transcription factor 1 mRNAs in anaplastic thyroid carcinoma tissues and cell lines. Oncology reports. 2007;18(3):715-20.
170. Liu X, Bishop J, Shan Y, Pai S, Liu D, Murugan AK, et al. Highly prevalent TERT promoter mutations in aggressive thyroid cancers. Endocrine-related cancer. 2013;20(4):603-10.
171. Landa I, Ganly I, Chan TA, Mitsutake N, Matsuse M, Ibrahimpasic T, et al. Frequent somatic TERT promoter mutations in thyroid cancer: higher prevalence in advanced forms of the disease. The Journal of clinical endocrinology and metabolism. 2013;98(9):E1562-6.
172. Liu T, Wang N, Cao J, Sofiadis A, Dinets A, Zedenius J, et al. The age- and shorter telomere-dependent TERT promoter mutation in follicular thyroid cell-derived carcinomas. Oncogene. 2013.
173. Melo M, da Rocha AG, Vinagre J, Batista R, Peixoto J, Tavares C, et al. TERT promoter mutations are a major indicator of poor outcome in differentiated thyroid carcinomas. The Journal of clinical endocrinology and metabolism. 2014;99(5):E754-65.
174. Wang N, Liu T, Sofiadis A, Juhlin CC, Zedenius J, Hoog A, et al. TERT promoter mutation as an early genetic event activating telomerase in follicular thyroid adenoma (FTA) and atypical FTA. Cancer. 2014.
175. Technologies™. L. TRIzol® Reagent http://www.lifetechnologies.com2012 [cited 2013].
176. Simoes AE, Pereira DM, Amaral JD, Nunes AF, Gomes SE, Rodrigues PM, et al. Efficient recovery of proteins from multiple source samples after TRIzol((R)) or TRIzol((R))LS RNA extraction and long-term storage. BMC genomics. 2013;14:181.
177. Vanecko S, Laskowski M, Sr. Studies of the specificity of deoxyribonuclease I. II. Hydrolysis of oligonucleotides carrying a monoesterified phosphate on carbon 3'. The Journal of biological chemistry. 1961;236:1135-40.
178. Marques AR, Espadinha C, Catarino AL, Moniz S, Pereira T, Sobrinho LG, et al. Expression of PAX8-PPAR gamma 1 rearrangements in both follicular thyroid carcinomas and adenomas. The Journal of clinical endocrinology and metabolism. 2002;87(8):3947-52.
179. Lima J, Trovisco V, Soares P, Maximo V, Magalhaes J, Salvatore G, et al. BRAF mutations are not a major event in post-Chernobyl childhood thyroid carcinomas. The Journal of clinical endocrinology and metabolism. 2004;89(9):4267-71.
180. Boichard A, Croux L, Al Ghuzlan A, Broutin S, Dupuy C, Leboulleux S, et al. Somatic RAS mutations occur in a large proportion of sporadic RET-negative medullary thyroid

carcinomas and extend to a previously unidentified exon. The Journal of clinical endocrinology and metabolism. 2012;97(10):E2031-5.
181. Moura MM, Cavaco BM, Pinto AE, Leite V. High prevalence of RAS mutations in RET-negative sporadic medullary thyroid carcinomas. The Journal of clinical endocrinology and metabolism. 2011;96(5):E863-8.
182. Ciampi R, Mian C, Fugazzola L, Cosci B, Romei C, Barollo S, et al. Evidence of a low prevalence of RAS mutations in a large medullary thyroid cancer series. Thyroid : official journal of the American Thyroid Association. 2013;23(1):50-7.
183. Rachakonda PS, Hosen I, de Verdier PJ, Fallah M, Heidenreich B, Ryk C, et al. TERT promoter mutations in bladder cancer affect patient survival and disease recurrence through modification by a common polymorphism. Proceedings of the National Academy of Sciences of the United States of America. 2013;110(43):17426-31.
184. Guerra A, Zeppa P, Bifulco M, Vitale M. Concomitant BRAF(V600E) mutation and RET/PTC rearrangement is a frequent occurrence in papillary thyroid carcinoma. Thyroid : official journal of the American Thyroid Association. 2014;24(2):254-9.
185. Unger K, Zitzelsberger H, Salvatore G, Santoro M, Bogdanova T, Braselmann H, et al. Heterogeneity in the distribution of RET/PTC rearrangements within individual post-Chernobyl papillary thyroid carcinomas. The Journal of clinical endocrinology and metabolism. 2004;89(9):4272-9.
186. Sobrinho-Simoes M, Preto A, Rocha AS, Castro P, Maximo V, Fonseca E, et al. Molecular pathology of well-differentiated thyroid carcinomas. Virchows Archiv : an international journal of pathology. 2005;447(5):787-93.
187. Soares P, Celestino R, Melo M, Fonseca E, Sobrinho-Simoes M. Prognostic biomarkers in thyroid cancer. Virchows Archiv : an international journal of pathology. 2014;464(3):333-46.
188. Leonard S, Logel J, Luthman D, Casanova M, Kirch D, Freedman R. Biological stability of mRNA isolated from human postmortem brain collections. Biological psychiatry. 1993;33(6):456-66.
189. Spruessel A, Steimann G, Jung M, Lee SA, Carr T, Fentz AK, et al. Tissue ischemia time affects gene and protein expression patterns within minutes following surgical tumor excision. BioTechniques. 2004;36(6):1030-7.

Supplementary tables

Table 14 - *Characterization of the genetic alterations in the malignant tumour samples, displaying the frequency and percentage within diagnosis (n=111).*

Diagnosis	*BRAF*			*NRAS*			*TERT*			
	WT	p.V600E	p.K601E	WT	p.Q61R	p.Q61K	WT	-124 C>T	-146 C>T	-150 C>T
PTC	**3** 42.9%	**4** 57.1%	**0** 0.0%	**7** 100.0%	**0** 0.0%	**0** 0.0%	**7** 100.0%	**0** 0.0%	**0** 0.0%	**0** 0.0%
cPTC	**28** 59.6%	**19** 40.4%	**0** 0.0%	**41** 87.2%	**6** 12.8%	**0** 0.0%	**46** 97.9%	**0** 0.0%	**0** 0.0%	**1** 2.1%
FVPTC	**23** 92.0%	**1** 4.0%	**1** 4.0%	**19** 76.0%	**4** 16.0%	**2** 8.0%	**24** 96.0%	**1** 4.0%	**0** 0.0%	**0** 0.0%
Diffuse sclerosing PTC	**4** 80.0%	**1** 20.0%	**0** 0.0%	**5** 100.0%	**0** 0.0%	**0** 0.0%	**5** 100.0%	**0** 0.0%	**0** 0.0%	**0** 0.0%
Tall cell PTC	**2** 100.0%	**0** 0.0%	**0** 0.0%	**2** 100.0%	**0** 0.0%	**0** 0.0%	**2** 100.0%	**0** 0.0%	**0** 0.0%	**0** 0.0%
SVPTC	**1** 100.0%	**0** 0.0%	**0** 0.0%	**1** 100.0%	**0** 0.0%	**0** 0.0%	**1** 100.0%	**0** 0.0%	**0** 0.0%	**0** 0.0%
Oncocytic PTC	**1** 100.0%	**0** 0.0%	**0** 0.0%	**1** 100.0%	**0** 0.0%	**0** 0.0%	**1** 100.0%	**0** 0.0%	**0** 0.0%	**0** 0.0%
Metastasis PTC	**2** 100.0%	**0** 0.0%	**0** 0.0%	**2** 100.0%	**0** 0.0%	**0** 0.0%	**2** 100.0%	**0** 0.0%	**0** 0.0%	**0** 0.0%
Metastasis FVPTC	**1** 100.0%	**0** 0.0%	**0** 0.0%	**0** 0.0%	**1** 100.0%	**0** 0.0%	**0** 0.0%	**0** 0.0%	**1** 100.0%	**0** 0.0%
FTC	**13** 100.0%	**0** 0.0%	**0** 0.0%	**9** 69.2%	**4** 30.8%	**0** 0.0%	**12** 92.3%	**1** 7.7%	**0** 0.0%	**0** 0.0%
Oncocytic FTC	**1** 100.0%	**0** 0.0%	**0** 0.0%	**1** 100.0%	**0** 0.0%	**0** 0.0%	**1** 100.0%	**0** 0.0%	**0** 0.0%	**0** 0.0%
PDTC	**3** 100.0%	**0** 0.0%	**0** 0.0%	**2** 66.7%	**0** 0.0%	**1** 33.3%	**3** 100.0%	**0** 0.0%	**0** 0.0%	**0** 0.0%
MTC	**3** 100.0%	**0** 0.0%	**0** 0.0%	**2** 66.7%	**0** 0.0%	**1** 33.3%	**3** 100.0%	**0** 0.0%	**0** 0.0%	**0** 0.0%
Total	**85** 76.6%	**25** 22.5%	**1** 0.9%	**92** 82.9%	**15** 13.5%	**4** 3.6%	**107** 96.4%	**2** 1.8%	**1** 0.9%	**1** 0.9%

Legend: PTC, papillary thyroid carcinoma; cPTC, classical papillary thyroid carcinoma; FVPTC, follicular thyroid carcinoma; diffuse sclerosing PTC, diffuse sclerosing variant of papillary thyroid carcinoma; tall cell PTC, tell cell variant of papillary thyroid carcinoma; SVPTC, solid variant of papillary thyroid carcinoma; oncocytic PTC, oncocytic variant of papillary thyroid carcinoma; metastasis PTC, metastasis of papillary thyroid carcinoma; metastasis FVPTC, metastasis of follicular variant of papillary thyroid carcinoma; FTC, follicular thyroid carcinoma; Oncocytic FTC, Oncocytic follicular thyroid carcinoma, PDTC, poorly differentiated thyroid carcinoma; MTC, medullary thyroid carcinoma; WT, wild type; *TERT* promoter polymorphism.

Table 15 - *Characterization of the genetic rearrangements, in the malignant tumour samples, displaying the frequency and percentage within diagnosis (n=109).*

Diagnostic	*RET/PTC1*		*RET/PTC3*		*PAX8-PPARɣ*	
	WT	**Rearranged**	**WT**	**Rearranged**	**WT**	**Rearranged**
PTC	**6** 85.7%	**1** 14.3%	**7** 100.0%	**0** 0.0%	**7** 100.0%	**0** 0.0%
Classic PTC	**38** 82.6%	**8** 17.4%	**46** 100.0%	**0** 0.0%	**46** 100.0%	**0** 0.0%
FVPTC	**23** 95.8%	**1** 4.2%	**23** 95.8%	**1** 4.2%	**23** 95.8%	**1** 4.2%
Diffuse sclerosing PTC	**5** 100.0%	**0** 0.0%	**4** 80.0%	**1** 20.0%	**5** 100.0%	**0** 0.0%
Tall cell PTC	**2** 100.0%	**0** 0.0%	**2** 100.0%	**0** 0.0%	**2** 100.0%	**0** 0.0%
SVPTC	**1** 100.0%	**0** 0.0%	**1** 100.0%	**0** 0.0%	**1** 100.0%	**0** 0.0%
Oncocytic PTC	**1** 100.0%	**0** 0.0%	**1** 100.0%	**0** 0.0%	**1** 100.0%	**0** 0.0%
Metastasis PTC	**1** 50.0%	**1** 50.0%	**2** 100.0%	**0** 0.0%	**2** 100.0%	**0** 0.0%
Metastasis FVPTC	**1** 100.0%	**0** 0.0%	**1** 100.0%	**0** 0.0%	**1** 100.0%	**0** 0.0%
FTC	**12** 92.3%	**1** 7.7%	**13** 100.0%	**0** 0.0%	**12** 92.3%	**1** 7.7%
Oncocytic FTC	**1** 100.0%	**0** 0.0%	**1** 100.0%	**0** 0.0%	**1** 100.0%	**0** 0.0%
PDTC	**3** 100.0%	**0** 0.0%	**3** 100.0%	**0** 0.0%	**3** 100.0%	**0** 0.0%
MTC	**3** 100.0%	**0** 0.0%	**3** 100.0%	**0** 0.0%	**3** 100.0%	**0** 0.0%
Total	**97** 89.0%	**12** 11.0%	**107** 96.4%	**2** 1.8%	**107** 96.4%	**2** 1.8%

Legend: PTC, papillary thyroid carcinoma; cPTC, classical papillary thyroid carcinoma; FVPTC, follicular thyroid carcinoma; diffuse sclerosing PTC, diffuse sclerosing variant of papillary thyroid carcinoma; tall cell PTC, tell cell variant of papillary thyroid carcinoma; solid SVPTC, solid variant of papillary thyroid carcinoma; oncocytic PTC, oncocytic variant of papillary thyroid carcinoma; metastasis PTC, metastasis of papillary thyroid carcinoma; metastasis FVPTC, metastasis of follicular variant of papillary thyroid carcinoma; FTC, follicular thyroid carcinoma; Oncocytic FTC, Oncocytic follicular thyroid carcinoma, PDTC, poorly differentiated thyroid carcinoma; MTC, medullary thyroid carcinoma; WT, wild type.

Table 16 - *Comparison between PTC without or with tumour capsule in relation to the variable multinodular goiter.*

Characteristics		Tumour capsule		p-value
		Absent	Present	
Multinodular goiter	Absent	**29** 74.4%	**21** 95.5%	**0.037**
	Present	**10** 25.6%	**1** 4.5%	

Legend: Statistical differences are considered as significant when p-value <0.05.

Table 17 - *Comparison between PTC without or with tumour capsule invasion in relation to the variable vascular invasion.*

Characteristics		Tumour capsule invasion		p-value
		Absent	Present	
Vascular invasion	Absent	**4** 80.0%	**2** 20.0%	**0,047**
	Present	**1** 20.0%	**8** 80.0%	

Legend: Statistical differences are considered as significant when p-value <0.05.

Table 18 *- Comparison between PTC without or with BRAF mutations in relation to the NRAS mutational status.*

Characteristics		*BRAF*		p-value
		WT	Mutated	
NRAS	WT	**33** 84.6%	**24** 100,0%	**0,048**
	Mutated	**6** 15.4%	**0** 0,0%	

Legend: Statistical differences are considered as significant when p-value <0.05; WT, wild type.

Table 19 *- Comparison between FVPTC without or with lymphocytic thyroiditis in relation to the variable multinodular goiter.*

Characteristics		Lymphocytic thyroiditis		p-value
		Absent	Present	
Multinodular goiter	Absent	**10** 58.8%	**8** 100.0%	**0.040**
	Present	**7** 41.2%	**0** 0.0%	

Legend: Statistical differences are considered as significant when p-value <0.05.

Table 20 - *Comparison between single and multiple FVPTC in relation to the variable tumour size.*

Characteristics	Number of tumours		p-value
	Single	Multiple	
Tumour size	**1.6**	**3.0**	**0.042**

Legend: Statistical differences are considered as significant when p-value <0.05.

Table 21*- Comparison between TERT promoter mutational status, considering all positive cases for the mutation, in relation to the variable age.*

Characteristics		*TERT promoter*		p-value
		WT	Mutated	
Age	**Mean**	**43.6**	**71.0**	**0.001**

Legend: Statistical differences are considered as significant when p-value <0.05; WT, wild type.