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The pathogenic role of mTOR pathway in papillary thyroid carcinoma and its impact on sodium iodide symporter (NIS) expression

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TESE DE DOUTORAMENTO APRESENTADA

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Ao abrigo do Art. 8º do Decreto-Lei nº 388/70, fazem parte integrante desta Dissertação os seguintes trabalhos já publicados, ou submetidos para publicação:

I - Tavares C., Coelho M.J., Melo M., Gaspar da Rocha A., Pestana A., Batista R., Salgado C., Eloy C., Ferreira L., Rios E., Sobrinho-Simões M. and Soares P. (2016) pmTOR is a marker of aggressiveness in papillary thyroid carcinomas. Surgery 160(6):1582-1590. doi: 10.1016/j.surg.2016.

II - Tavares C., Eloy C., Melo M., Gaspar da Rocha A., Pestana A., Batista R., Ferreira LB, Rios E., Sobrinho Simões M. and Soares P. (2017) mTOR pathway in papillary thyroid carcinoma: different contributions of mTORC1 and mTORC2 complexes to tumor behavior and *SLC5A5*mRNA expression. *Em preparação.*

III - Tavares C., Coelho M.J., Eloy C., Melo M., Gaspar da Rocha A., Pestana A., Batista R., Ferreira L.B., Rios E., Selmi-Ruby S., Cavadas B., Pereira L., Sobrinho-Simões M. and Soares P. (2017) The usefulness of the study of sodium iodide symporter expression in thyroid primary tumors. *Submetido para publicação.*

O seguinte artigo não faz parte do corpo principal da tese, mas é parte integrante da mesma, tendo sido utilizado na sua Introdução e Discussão:

Apêndice I – Tavares C., Melo M., Cameselle-Teijeiro J.M., Soares P., Sobrinho-Simões M. (2016) ENDOCRINE TUMOURS: Genetic predictors of thyroid cancer outcome. European Journal of Endocrinology 174(4):R117-26. doi: 10.1530/EJE-15-0605.

Em cumprimento com o disposto no Decreto-Lei nº 388/70, declara que participou ativamente na recolha e estudo do material incluído em todos os trabalhos, redigiu os artigos I, II, III e parte do Apêndice I. Esta Dissertação inclui também resultados de trabalhos não publicados.

Vou viver
até quando eu não sei
que me importa o que serei
quero é viver

Amanhã, espero sempre um amanhã
e acredito que será
mais um prazer

e a vida é sempre uma curiosidade
que me desperta com a idade
interessa-me o que está para vir
a vida em mim é sempre uma certeza
que nasce da minha riqueza
do meu prazer em descobrir

encontrar, renovar, vou fugir ou repetir (…)

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Resumo

O cancro da tiroide (CT) é a neoplasia endócrina mais comum. A grande maioria dos CT deriva das células foliculares tiroideias e mantém um certo grau de diferenciação, sendo denominado, nesses casos, carcinoma diferenciado da tiroide (CDT). Os CDT compreendem os carcinomas papilares (CPT) (~85% dos casos de CDT) e os carcinomas foliculares (~15% dos casos de CDT).

Os doentes com CDT têm, na sua grande maioria, um bom prognóstico. O tratamento faz-se por cirurgia seguida por terapia ablativa com iodo radioativo (^{131}I) para destruição de possíveis remanescentes e/ou metástases. A eficácia da radioterapia com iodo deve-se, pelo menos em parte, à presença e à função preservada do transportador de sódio e iodo (NIS), codificado pelo gene *SLC5A5*, e localizado na membrana plasmática das células tumorais. O NIS capta o ^{131}I para o interior das células tumorais, afetando minimamente as estruturas adjacentes. É uma radioterapia dirigida muito eficiente, que contribui para o bom prognóstico dos doentes com CDT.

Infelizmente, um pequeno grupo de doentes com CDT desenvolve recidivas tumorais que deixam de captar o ^{131}I (cerca de 26-60% dos doentes com recidivas deixam de captar o ^{131}I), tornando-se resistentes à terapia. A perda de expressão/função do NIS é o mecanismo molecular melhor conhecido como "contribuinte" para a resistência à terapia com ^{131}I. Esse grupo de doentes representa um verdadeiro desafio, pois como a sua identificação não é possível aquando do diagnóstico, todos os casos de CDT são tratados da mesma forma, e eventualmente sobretratados. É por isso premente a identificação de biomarcadores que permitam um reconhecimento precoce destes casos.

A via do mTOR encontra-se sobreativada numa grande variedade de neoplasias humanas, estando por vezes associada a maior agressividade tumoral e pior prognóstico. Uma vez ativado, o mTOR pode dar origem à formação de dois complexos distintos: o mTORC1 e o mTORC2, cada um com efetores diferentes e com funções biológicas distintas. A via do mTOR encontra-se também sobreativada no CT mas as consequências biológicas de tal sobreativação permanecem

desconhecidas. Além de sobreativada, a via do mTOR parece desempenhar também um papel na regulação da expressão do NIS.

Vários estudos têm abordado a expressão do NIS (mRNA e proteína) em diferentes tecidos tiroideus. Os tumores apresentam uma menor expressão do gene *SLC5A5* do que o tecido tiroideu normal. No entanto, a respetiva proteína parece estar em maior quantidade no tumor embora localizada no citoplasma em vez da localização habitual na membrana citoplasmática. Os mecanismos moleculares que conduzem à perda da expressão/função do NIS permanecem pouco esclarecidos, assim como a utilidade que a avaliação da expressão do NIS (nos tumores primários) pode ter para prever a resposta à terapia com ^{131}Ie/ou o comportamento tumoral.

Neste trabalho pretendemos encontrar novos marcadores de agressividade tumoral e de resistência à terapia com ^{131}I, com o objetivo de procurar estratificar melhor os pacientes com CDT. Para tal, caracterizámos a via do mTOR, através da avaliação da expressão do pmTOR Ser2448, pS6 Ser235/236 (efetor do complexo mTORC1) e pAKT Ser473 (efetor do complexo mTORC2) numa grande série de CPT. Avaliámos também a expressão do NIS (mRNA e proteína) numa grande série de CDT. Em seguida, avaliámos possíveis associações entre a expressão desses dois marcadores e as características clinocopatológicas e moleculares dos casos, o seu prognóstico e resistência à terapia com ^{131}I. Para validar os nossos resultados, analisámos ainda a expressão do gene *SLC5A5* numa serie de 378 CPT, através de dados recolhidos da base de dados do projeto denominado "The Cancer Genome Atlas".

Os nossos resultados demonstraram que o pmTOR é um marcador de agressividade em CPT, que pode eventualmente estar associado à resistência à terapia com ^{131}I (maior número de terapias com ^{131}I e menor expressão do gene *SLC5A5*). A expressão do pS6 foi associada a características clinicopatológicas de menor agressividade e à ausência da mutação do gene *BRAF*. Não encontrámos correlação entre a expressão do pS6 e do pmTOR. Observámos que a expressão do pAKT se correlacionava positivamente com a expressão do pmTOR, que era significativamente maior nos CPT com mutação do gene *BRAF*V600E; observámos mais ainda que a translocação nuclear do pAKT se associava significativamente à presença de metástases à distância.

Uma vez que também estávamos interessados no impacto que a via do mTOR poderia ter na expressão do gene *SLC5A5*, procedemos ao bloqueio farmacológico do complexo mTORC1, e dos complexos mTORC1 e C2, com rapamicina e Torin 2, respetivamente. Observámos que o bloqueio do complexo mTORC2 desempenha uma função na regulação da expressão do gene *SLC5A5*; tendo o seu bloqueio promovido a re-expressão deste gene.

Observámos que a baixa expressão do gene *SLC5A5* se associou a características patológicas de maior agressividade e de pior prognóstico. A expressão proteica do NIS não se associou, na nossa série, nem com prognóstico, nem com resposta à terapia com ^{131}I, o que leva a concluir que a avaliação dessa expressão tem pouco valor prático. Observámos também que o contexto genético tumoral (*RAS*, *BRAF* e *TERT*p) tem um grande impacto na expressão do gene *SLC5A5*e na localização membranar do NIS. Os CPT não portadores das mutações estudadas apresentavam uma expressão do gene *SLC5A5* significativamente maior comparativamente àqueles que continham pelo menos uma. As mutações do gene *RAS* foram aquelas que demonstraram causar o menor impacto na expressão do gene *SLC5A5*, seguidas pelas do *BRAF* e do *TERT*p, respetivamente.

Concluindo, nesta tese demonstrámos que o pmTOR é um marcador de agressividade tumoral e de provável resistência à terapia em doentes com CPT. As suas ações parecem ser mediadas pelo efetor do complexo mTORC2, o pAKT cuja translocação nuclear se encontra associada a metastização à distância. Verificámos também que a inibição do complexo mTORC2 é capaz de aumentar os níveis de expressão do gene *SLC5A5*. Estes resultados chamam a atenção para a via do mTOR como potencial alvo terapêutico para CPT metastáticos e/ou refratários à terapia com ^{131}I. Adicionalmente, observámos que a baixa expressão do gene *SLC5A5* no tumor primário se associa a maior agressividade tumoral e pior prognóstico; estes achados sugerem que a referida expressão poderá constituir um novo marcador para estratificação do risco/prognóstico dos doentes com CDT.

Abstract

Thyroid cancer (TC) is the most prevalent endocrine malignancy. The vast majority of TC derives from follicular cells and maintains a certain degree of differentiation, being in that case denominated differentiated thyroid carcinoma (DTC). DTC can be further divided in papillary thyroid carcinoma (PTC) (~85% of DTC cases) and follicular thyroid carcinoma (FTC) (~15%of DTC cases).

DTCs carry, in general, a very good prognosis. Treatment is based on surgery followed by radioactive iodine (RAI) ablation of tumor remnants and/or metastases. The effectiveness of this radiotherapy depends, at least in part, on the presence and preserved function of sodium iodide symporter (NIS), codified by the *SLC5A5* gene, in the membrane of TC cells. NIS uptakes RAI into the tumor cells, while the adjacent structures remain unaltered. It is a very efficient, targeted radiotherapy that contributes to the very good prognosis of most patients with DTC. Unfortunately, a subgroup of DTC patients develops tumor recurrences; in this setting the tumor tissue loses the ability to uptake RAI (~ 26-60% of the patients with recurrent disease) and become resistant to RAI therapy. The loss of NIS expression/function is the major molecular mechanism contributing to RAI refractoriness. This group of patients represents a real challenge because it is still not possible to predict which DTC patients will develop recurrent and/or refractory disease. It is crucial to progress in the identification of biomarkers that allow the early recognition of such patients in order to turn the intensity of RAI therapy more appropriate and avoid the overtreatment of many DTC patients.

mTOR pathway is overactivated in a great variety of human neoplasms, being occasionally associated with tumor aggressiveness and worse prognosis. Once activated, mTOR can give rise to the assembly of two distinct complexes: mTORC1 and mTORC2, with distinct downstream effectors and functions. mTOR pathway is also activated in TC, but the biological consequences of such activation remain unknown. Besides being overactivated, mTOR pathway seems to play a role on NIS expression regulation.

Several studies have addressed the issue of NIS expression (mRNA and protein) in different thyroid tissues, reporting its downregulation or mistargeting to the membrane in tumors compared to normal thyroid. The molecular mechanisms that contribute to that downregulation/loss of function are not fully understood, and the impact of NIS expression in thyroid primary tumors in terms of predicting RAI therapy response and/or tumor behavior remains unclarified.

In an attempt to find new markers of aggressiveness and therapy resistance in primary DTCs, and to contribute to a better stratification of the patients, we characterized the mTOR pathway status through the expression of pmTOR Ser2448, pS6 (Ser235/236 mTORC1 downstream effector) and pAKT (Ser473 mTORC2 downstream effector) in a large series of PTCs. Furthermore, we also addressed NIS (mRNA and protein expression) in a large series of DTCs. Having these data as background, we explored possible associations between the expression of those markers with clinicopathological and molecular features, prognosis and response to RAI therapy. To validate our results, we also studied the *SLC5A5* mRNA expression from 378 PTCs, retrieved from The Cancer Genome Atlas.

Our findings demonstrated that pmTOR is a marker of aggressiveness in PTCs, being particularly associated with distant metastization, and possibly with RAI therapy resistance (low *SLC5A5* mRNA expression and higher number of RAI therapies). The expression of pS6 was associated with less aggressive pathological features and with *BRAF*WT status. There was no significant correlation between pmTOR and pS6 expression. At variance with this, the expression of pAKT was positively correlated with pmTOR expression, significantly increased in *BRAF*V600E mutated PTCs and its nuclear translocation was associated with distant metastization.

Since we were also interested in the impact of the mTOR pathway in *SLC5A5* mRNA expression, we blocked pharmacologically mTORC1, and mTORC1 and C2 complexes with rapamycin and Torin2, respectively, in a PTC derived cell line. We observed that mTORC2 complex plays a role in *SLC5A5* mRNA expression regulation: its inhibition increased substantially *SLC5A5* mRNA expression. We further observed that a lower *SLC5A5* mRNA expression was associated with aggressive pathological features and worse prognosis.

NIS protein expression was not significantly associated with prognosis or RAI therapy response, thus being in our opinion, of limited value.

Finally, we also observed that the genetic background (*RAS*, *BRAF* and *TERT*p mutation) is of major importance to both *SLC5A5* mRNA expression and NIS targeting to the membrane. PTCs wild type for the aforementioned mutations presented higher *SLC5A5* mRNA expression compared to those harboring any mutation. The *RAS* mutation presented the lower impact on *SLC5A5* mRNA expression, followed by *BRAF* and *TERT*p mutations, respectively.

In conclusion, we demonstrated that pmTOR pathway is a marker of metastatic and, probably, RAI refractory PTCs. Its actions seem to be mediated by mTORC2 downstream effector pAKT whose nuclear translocation was associated with distant metastization and whose inhibition caused an increase of *SLC5A5* mRNA expression. These results single out the mTOR pathway as an attractive therapeutic target for advanced refractory PTC treatment. The observation that *SLC5A5* mRNA expression in primary tumors was associated with higher tumor aggressiveness and worse prognosis, suggests that such expression may be useful in DTC patient risk/prognostic stratification.

List of abbreviations

^{131}I – iodine 131

4E-BP1 - eukaryotic translation initiation factor 4E-binding protein 1

AKT –v-akt murine thymoma viral oncogene homolog

ATA – american thyroid association

ATC – anaplastic thyroid carcinoma

ATP – adenosine triphosphate

BRAF – B-Raf proto-oncogene, serine/threonine kinase

cAMP - cyclic adenosine monophosphate

cPTC - classic PTC

CT - computed tomography

Deptor - DEP domain-containing mTOR-interacting protein

DIT - diiodotyrosine

DMSO - dimethyl sulfoxide

DTC - differentiated thyroid carcinoma

DUOX 2 - dual oxidase 2

EFVPTC – encapsulated variant of PTC

eIF4E - eukaryotic translation initiation factor 4E

EMT - epithelial-mesenchymal transition

ER – endoplasmatic reticulum

ERK - extracellular regulated MAP kinase

ET – extrathyroidal extension

ETA – european thyroid association

FDA - food and drug administration

FNA - fine-needle aspiration

FTA – follicular thyroid adenoma

FTC - follicular thyroid carcinoma

FVPTC – follicular variant of PTC

GTP - guanosine triphosphate

H2O2 – hydrogen peroxide

HDACs - histone deacetylases

Hsp70 - heat shock protein 70-alpha

MAPK - mitogen-activated protein kinase

MEK - mitogen-activated protein kinase kinase

MIT - monoiodotyrosine

mLST8 - mammalian lethal with SEC13 protein 8

MRI - magnetic resonance imaging

mRNA – messenger RNA

mSin1 - mammalian stress activated protein kinase interacting protein 1

MTC - medullary thyroid carcinoma

mTOR - mammalian target of rapamycin

mTORC1/2 - mTOR complex 1/2

NADPH - nicotinamide adenine dinucleotide phosphate

NIS – sodium iodide sympoter

NRAS - neuroblastoma RAS viral (v-ras) oncogene homologue

PDTC - poorly differentiated thyroid carcinoma

PET - positron emission tomography

PI3K - phosphatidylinositol 3-kinase

PKC α - protein kinase Cα

PRAS40 - proline rich Akt substrate 40

PRR5 / Protor - proline rich protein 5 / protein observed with rictor

PTC - papillary thyroid carcinoma

PTEN - phosphatase and tensin homologue deleted on chromosome ten

RAF -raf-1 proto-oncogene, serine/threonine kinase

RAI - radioactive iodine

Raptor - regulatory associated protein of mTOR

RAS - rat sarcoma virus oncogene

RET-PTC – rearrangement of the RET oncogene

Rheb - ras homolog enriched in brain

Rictor - rapamycin insensitive companion of mTOR

RSK - p90 ribosomal S6 kinase

S6 - 40S ribosomal protein S6

S6K1 - ribosomal protein S6 kinase beta-1

S6K2 - ribosomal protein S6 kinase beta-2

SLC5A5 - solute carrier family 5 member 5

T3 - triiodothyronine

T4 - thyroxine

TC- thyroid cancer

TERT - telomerase reverse transcriptase

Tg - thyroglobulin

TGF-β - transforming growth factor-beta

TKI - tyrosine kinase inhibitor

TNM - tumor, node, metastases

TPO - thyroperoxidase

TRH - thyrotropin-releasing hormone

TSC ½ - tuberous sclerosis complex 1/2

TSH - thyroid stimulating hormone

TSHR - thyroid stimulating hormone receptor

UICC/AJCC - union for international cancer control/american joint committee on cancer

WBS - whole body scan

WT - wild-type

List of figures and tables

Chapter 1. Introduction

1. Thyroid physiology

1.1 Thyroid gland function, regulation and constitution

Thyroid gland is located in the anterior neck and consists of two lobes connected by a band of thyroid tissue or isthmus, which lies just below the cricoid cartilage of the larynx[1-3]. The main function of the thyroid gland is to produce hormones: T4 (thyroxine or tetraiodothyronine) and T3(triiodothyronine), that regulate the differentiation of the central nervous system, body growth[4], and pathways of body energy and intermediary metabolism[1-5].

The production of thyroid hormones is a complex process that occurs in line with the body needs; the hypothalamus produces and releases thyrotropin-releasing hormone (TRH) that binds to receptors on the plasma membrane of thyrotrophs of the pituitary gland which stimulates the secretion of thyroid stimulating hormone (TSH) into the blood. Once in the bloodstream, TSH will act on the thyroid gland, increasing the rate of thyroid hormone secretion[4]. This process is tightly regulated; thyroid hormones exert a direct negative-feedback effect on both hypothalamus (decreasing TRH release) and in the pituitary (reducing the sensitivity of the thyrotrophs to TRH), consequently TSH synthesis decreases, and the levels of T3 and T4 fall. This negative feed-back control system is part of the hypothalamic-pituitary- thyroid axis[4] (Figure 1).

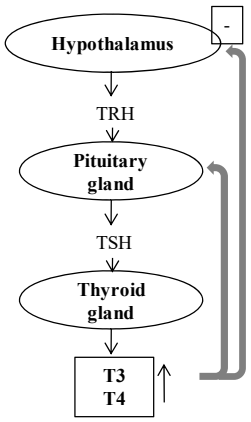

Figure1. Hypothalamic-pituitary-thyroid axis. T3 and T4 high levels activate a negative feedback loop causing a decrease of TRH production and consequently a decrease of thyroid hormones synthesis.

Thyroid gland is constituted by spherical structures called follicles, which in its turn are composed by follicular cells disposed side by side forming a structure that encloses a gel-like substance called colloid in the lumen of the follicle. In addition to the follicular cells, thyroid gland is also constituted by another endocrine cell type, the parafollicular cells, located in the wall of the thyroid follicle, inside the basal lamina (Figure 2). The follicular cells are the ones responsible for thyroid hormone (T3 and T4) synthesis and, parafollicular cells produce and secrete the hormone calcitonin, involved on calcium metabolism[1-3].

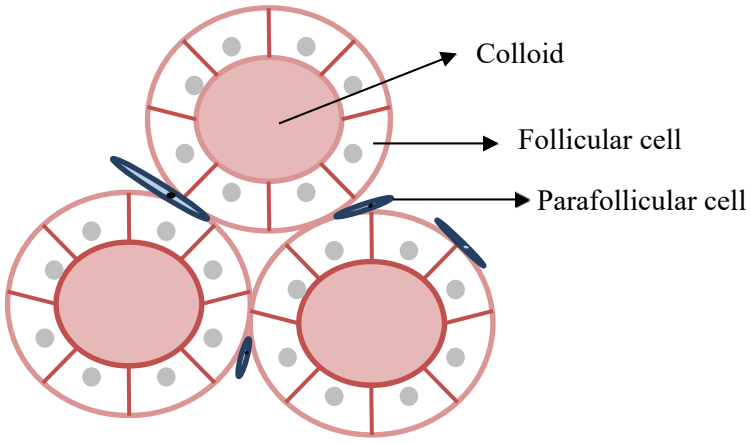

Figure 2. Schematic representation of normal thyroid histology. Thyroid gland is composed by follicular cells, enclosing the colloid, and by parafollicular cells.

1.2 Production of thyroid hormones

In order to produce thyroid hormones, the follicular cells need two "raw materials": thyroglobulin (Tg) and iodine; and also a complex network of membrane transporters and enzymes[1-3, 5]: Once thyroid gland is stimulated by TSH, all these components interact through orchestrated processes, culminating with the production of the hormones.

1.2.1 Thyroglobulin synthesis

Tg is a very large glycoprotein (600KDa) synthesized on the rough endoplasmatic reticulum (ER) of follicular cells and that undergo dimerization and glycosylation in the smooth ER. The completed glycoprotein is packaged into vesicles by the Golgi apparatus[1]. It accounts for approximately half of the protein content of the thyroid gland. Each molecule of Tg contains about

70 tyrosine residues that are the major substrates that combine with iodine[1, 2]. The vesicles containing Tg migrate to the apical membrane of the follicular cell and fuse with it, shedding Tg into the colloid[3].

1.2.2 Iodide trapping

The only source of iodine is the dietary intake, being rapidly absorbed into the bloodstream[3], through the small intestine[5]. It is then uptaken into the interior of the follicular cell against gradient; it is 30x more concentrated in the follicular cell compared to the blood. The capacity of the thyroid gland to accumulate iodine under physiological conditions, was first described in 1896, back then a I^- transporter was inferred[6]. It took 100 years to understand that iodine enters into the thyroid follicular cell through a specialized intrinsic plasma glycoprotein named sodium iodine symporter (Na^+/I^- symporter-NIS), codified by the *SLC5A5* gene[7].

NIS is located in the basolateral membrane of the follicular cells; it mediates iodine transport by using the energy released by the inward translocation of $2Na^+$ions down to its electrochemical gradient. The maintenance of the Na^+ gradient acting as the driving force is insured by Na^+-K^+-ATPase [3, 5]chanels.

1.2.2.1 NIS in a physiological context

The detailed molecular characterization of NIS started when Dai et al.,[7] isolated the cDNA encoding rat NIS (rNIS) by expression cloning in X. laevis oocytes, using cDNA libraries derived from FTRL-5 cells (a highly functional rat thyroid derived cell line)[7]. Hoping that human NIS (hNIS) would be highly similar to rNIS, Smanik et al.,[8] using primer for rNIS, identified a cDNA clone encoding hNIS, constituted by an open reading frame of 1929 nucleotides, encoding a protein of 643 aminoacids (approximately 70-90KDa)[8]. Later, Smanik et al.,[9] examined the expression, exon-intron organization and chromosome mapping of hNIS: fifteen exons encoding hNIS were mapped to chromosome 19p12-13.2[9]. hNIS exhibits 84% identity and 93% similarity to rNIS[10]. cDNAs encoding NIS have also been isolated from other two different species, pig[11] and mouse[12]. Mouse[12] and rat[7] NIS contain 618 aminoacids while pig[11] and human[8] NIS display 643 aminoacids. There is a high sequence identity between species[10].

The current secondary structure model for NIS proposes 13 transmembrane segments, the NH2 terminal in the extracellular face and the COOH terminal in the cytosol[13]. Being a glycoprotein, NIS is glycosylated at Asn residues 225, 485 and 497 (of the rat sequence), however the role of glycosylation on NIS targeting and function is not consensual, some authors claim that glycosylation is not essential for NIS stability, targeting or function[13], but a recent study showed that NIS glycosylation can modulate both NIS targeting and function[14]. Several phosphorylation sites have been identified in the molecule, only three charged residues were predicted to lie within transmembrane segments[15].

NIS belongs to the solute carrier family 5, which includes the high affinity Na+-glucose co-transporter family (*SLC5A1*), the low Na+-glucose co-transporter (*SLC5A2*), the Na+-myoinositol transporter (*SLC5A3*), the Na+-dependent proline symporter (*SLC5A4*) and the Na+-dependent multivitamin transporter (*SLC5A6*)[10].

There are many players that contribute to NIS expression regulation in normal thyroid and, in the following section we present the ones that are better studied: TSH, iodine and follicular cell polarization.

TSH is the main regulator of NIS transcription in normal thyroid cells[10, 15, 16], modulating its expression and function trough transcriptional and post transcriptional events[17, 18]: TSH increases NIS transcription[19], modulates its half-life (5 days in the presence of TSH and 3 days in its absence[20]) and also regulates its targeting and/or retention in the plasma membrane and its phosphorylation status[13, 20, 21].TSH links to TSH receptor (TSHR) activating adenylyl cyclase through Gs-protein, resulting in the production of cyclic AMP (cAMP) which contributes at least in part, to NIS transcription activation[13, 19, 22, 23].

NIS gene expression regulation can take place at two different sites; NIS proximal promoter (NIS_PP) and the NIS upstream enhancer (NUE)[10, 16]. NUE involves the most relevant aspects of NIS regulation[24]. Different transcription factors are also involved on NIS transcription regulation, NK2 homeobox 1 (NKX2-1) previously named thyroid transcription factor (TTF1)[25] and paired domain transcription factor-8 (Pax8)[23].

NUE contains two Pax8 and two NKX2-1 binding sites (that do not contribute to NIS transcription) and a degenerate CRE (cAMP responsive element) sequence. For total activation of the NUE, both PAX8 and unidentified CRE-like binding factor (CRE-LBF) acts synergically to obtain full TSH-cAMP-dependent transcription[23]. NIS_PP has a binding site for NKX2-1 and for another transcription factor named NIS TSH-responsive factor (NTF-1)[26].

TSH stimulates both NIS_PP and NUE activity[23, 25, 27]. cAMP stimulation of NUE usually occurs through protein kinase A (PKA)[27], however, NUE is also able to mediate cAMP-dependent transcription by a novel PKA independent mechanism[23].More recently, forkhead transcription factor (FoxE1), previously known by thyroid transcription factor 2 (TTF-2) was also reported to be a transcription factor that can stimulate NIS transcription via NUE[28].

TSH can also regulate NKX2-1[29], Pax8[30] and FoxE1[31] expression and though contribute to NIS expression regulation by other mechanisms rather than cAMP.

The other main regulator of NIS expression is iodine itself[10]. When I^- levels reach a high threshold occurs an impairment of the organic I^- binding and thyroid hormone synthesis, this phenomenon was observed the first time in 1948 and is known as the acute Wolff and Chaikoff effect[32]. Approximately two days later, even in the presence of high plasma I^- concentration, occurs an "escape" from the acute effect, and consequently, the level of I^-organification is restored and normal hormone biosynthesis is established[33]. This phenomenon is an intrinsic highly auto regulatory system that protects the thyroid gland from high doses of iodide, and also ensures a correct iodide uptake for thyroid hormone biosynthesis. Further studies revealed that the molecular basis of the "escape" is the decrease of NIS expression, which is mediated, at least in part by a transcriptional[34] and also postranscriptional[35] mechanisms. Recently, Serrano-Nascimento et al[36] proposed that high intracellular I^- levels downregulate NIS expression by repressing Pax8 and p65 (NF-κB subunit known to increase NIS transcriptional activity[37]). Moreover their results indicated that excess of I^- repressed NIS expression through ROS-induced activation of PI3K/Akt signaling pathway[36]. Other authors have previously reported that I^-excess triggered an increase on ROS production[38]

Another factor that seem to control NIS activity is the state of cellular polarization: TSH induces NIS expression both in monolayer cells and in follicle-forming human primary culture thyrocytes, but significant stimulation of the I- uptake was only observed in the follicles[39]. A more recent study suggested that TSH activation of NIS gene transcription might involve, in addition to others, a regulatory factor(s) whose synthesis and/or activity are triggered by cell-cell interaction(s)[40]. A correct spacial organization of the thyroid seems critical to its function.

1.2.3 Iodine organification

Once in the follicular cell, iodine is dropped into the colloid[2,3], probably by another channel called pendrin[3]. The next step in the formation of thyroid hormones is the iodination of Tg, which is mediated by the enzyme thyroid peroxidase (TPO). TPO is located in the apical membrane of the follicular cell or attached to it. It catalyzes the oxidation of iodine (I^-) to iodide (I^0), in the presence of hydrogen peroxide (H2O2) and then incorporates it into specific tyrosine amino acids from Tg[1-3, 5].

The H2O2 is essential for TPO activity and consequently for thyroid hormone production. In thyroid, H2O2 production is assured by an enzyme named dual oxidase 2 (DUOX2), a membrane-bound NADPH-dependent flavoprotein, also present in the apical membrane of the follicular cell, next to TPO[5]. Excess H2O2 not involved in the oxidation of iodide may act as mutagenic or carcinogenic. Selenium containing glutathione peroxidase is therefore typically upregulated to provide protection from oxidative damage[41].

1.2.4 Conjugation

The iodination of Tg leads to the formation of monoiodotyrosine (MIT) residues, which remains in peptide linkage in the Tg structure. A second iodine atom may be added to a MIT residue by this same enzymatic process, forming a diiodotyrosine (DIT) residue. The final step in hormone synthesis it is called conjugation: it consists in the coupling of two neighboring iodotyrosyl residues to form iodothyronine: two DIT monomers form T4; one DIT and one MIT form T3[1-3, 5].

1.2.5 Proteolysis

When the thyroid gland is stimulated to secrete thyroid hormones, pinocytosis occurs at the apical membrane of follicular cells. Briefly the apical membrane form pseudopods in to the lumen embracing little quantities of colloid, forming endocytic vesicles (colloid droplets) that further migrate towards the basal membrane of the cell. In this path, colloid droplets merge with lysosomes full of proteases that digest the Tg molecules and release T3 and T4 in free form. These then diffuse through the base of the thyroid cell into the surrounding capillaries.

2. Thyroid cancer

2.1 Thyroid cancer epidemiology

Thyroid cancer (TC) incidence has been rising all over the years. In the United States of America (USA) the incidence increased gradually from 4.9 per 100 000 cases in 1975 to 15.07 per 100 000 cases in 2013[42]. This trend has also been observed in other countries across Europe, Asia, Oceania and South America[43]. The incidence was always higher in women[42, 43]. This increase in the incidence rate was not accompanied by an increase in the mortality rate, that remained stable throughout the years in both sexes[42]. Despite the steady worldwide increase, the incidence of TC remains relatively uncommon: 16.5 per 100000 in the USA and 7 per 100000 in Europe with the mortality varying between 0.6 and 0.8 per 100000 inhabitants, respectively (in 2012)[44].

This significant increment in TC incidence has been largely attributed to the more usual use of ultrasonography, although environmental factors may also be important. A study reported that the rising incidence of TC was predominantly due to the increased detection of small papillary cancers. This trend combined with the stable overall mortality suggest that the increasing incidence reflects increased detection of subclinical disease, rather than a true uprising in the occurrence of TC[45]. Furthermore, it was previously reported that small papillary carcinomas were a common finding at autopsy, reaching a frequency of 36% [46], suggesting that there is a large reservoir of small papillary carcinomas, without clinical presentation during life, but can be

uncovered by ultrasonography or other screening techniques[47]. Nevertheless another study analyzed the incidence rates of differentiated thyroid carcinoma of all sizes between 1988 and 2005 and observed an increment of incidence across all tumor sizes suggesting that increased diagnostic scrutiny may not be the sole explanation, environmental factors may also be important[48].

In Portugal, the incidence of TC has also been increasing in both sexes. The incidence rate in women is higher compared to men and is the highest compared to other European countries and even the world. This increased incidence is predominantly due to women of the north of the country. On the contrary, the mortality rate has decreased for women and slightly increased for men (with a greater increase in the south). These trends combined with an overall low mortality and high 5-year relative survival, raised some questions about the possible impact that an over-diagnosis might be causing[49].

2.2 Thyroid cancer diagnosis

The diagnosis of TC occurs mainly between 45-54 years (median 51). According to their anatomical location and size, thyroid nodules (a discrete lesion within the thyroid gland that is radiologically distinct from the surrounding thyroid parenchyma) can be noted by the patient and/or doctor or incidentally "found" in a routine ultrasonography[50].

The 2015 American Thyroid Association (ATA) Guidelines recommend thyroid nodule diagnostic fine needle aspiration (FNA) in the following cases:

A) Nodules > 1cm in greatest dimension with high suspicion sonographic pattern;

B) Nodules > 1 cm in greatest dimension with intermediate suspicion sonographic;

(C) Nodules > 1.5cm in greatest dimension with low suspicion sonographic pattern.

Nodules ≤ 1cm that course with clinical symptoms and/or lymphadenopathy also require further evaluation[50].

The FNA will be evaluated and classified according to the Bethesda system. This system recognizes six diagnostic categories (DC), and for each one provides estimation of cancer risk and proposes a clinical management: DC1 (non-diagnostic/unsatisfactory) the FNA should be

repeated; DC2 (benign) the patient must be under surveillance; DC3 (atypia of undetermined significance/follicular lesion of undetermined significance) and DC4 (follicular neoplasm or suspicious of follicular neoplasm) patient needs surveillance or surgery, depending on the clinical risk factors, ultasonography patterns, genetic testing and patient preferences); DC5 (suspicious of malignancy) and DC6 (malignant) patients should be subjected to surgery[50, 51].

2.3 Thyroid cancer histology

TC can derive from follicular cells (98-99% of cases) and from parafollicular cells (that originate medullary carcinoma, a rare type of TC (1-2% of cases)[52] that will not be addressed in this thesis). Tumors derived from follicular cells can be divided in three groups, according to their degree of differentiation: differentiated thyroid carcinomas (DTC) accounting for more than 97% of cases, poorly differentiated thyroid carcinomas (PDTC) and anaplastic thyroid carcinoma (ATC) that together represent less than 3% of the cases[50, 52, 53].

The DTC group can be further divided in papillary thyroid carcinoma (PTC), the most common DTC subtype (~85% of the cases) and follicular thyroid carcinoma (FTC) that represents ~15% of DTC cases[50, 54].

There are some molecular evidences indicating that DTC can go through a process of dedifferentiation and give rise to PDTC and ultimately to ATC, nonetheless, the PDTC and ATC can be originated as *de novo* TCs[55-58].

2.4 Differentiated thyroid carcinoma

2.4.1 Incidence, types, subtypes, and histological characteristics

PTC is the most common thyroid tumor, representing 80-85% of all TC. Macroscopically, the lesions are firm, usually white and with an invasive appeearance[59].

Microscopically, neoplastic cells are organized in papillae, that contain a core of fibrovascular (occasionally only fibrous) tissue[59, 60]. The diagnosis of PTC depends almost exclusively on the identification of the typical nuclei: large, irregular, clear and grooved[59, 61, 62]. Additionally, it is also frequently observed other morphological features such as unencapsulation,

prominent stromal reaction, psammoma bodies, elongated shape of the follicles and variegated appearance[61, 62]. Mitoses are exceptionally unusual[59, 63]. PTCs invade lymphatic vessels, leading to multifocal lesions and to regional lymph node metastases. The vascular invasion is rare and only 5-7% of PTCs develop distant metastases[59, 63]. PTCs that are composed totally or in part by papillae besides the aforementioned nuclear features, are classified as classic PTC (cPTC), comprising the most frequent histotype of PTC (~80% of the cases).

The follicular variant of PTC (FVPTC) is the second most common variant, being found in ~20% of patients with PTC[64]. The first histological description of FVPTC was by Lindsay in 1960[65], followed by Chen in 1977[66] and Rosai in 1983[67]. It is characterized as a tumor possessing the nuclear features typical of PTC, but a follicular growth pattern instead of papillae.

FVPTC presents several diagnostic and management challenges, they can be divided in: infiltrative (or non encapsulated) and in encapsulated follicular variant of PTC (EFVPTC). In the first group, the diagnosis is easy, there is no capsule and an invasive pattern can be observed. The EFVPTC diagnosis is more challenging, being encapsulated and harboring follicular architecture, they may be mistaken for follicular adenoma or follicular carcinoma[62]. The EFVPTC is diagnosed as PTC if the nuclear features are diffusely present throughout the tumor[59], which in many cases is subjective, leading to high interobserver variability[59, 68], so the most controversial lesions in FVPTC are those encapsulated, without invasion and/or multifocal and/or with imperfect nuclear features[59].

Noninvasive EFVPTC display a particularly indolent behavior (only a few cases will behave in a clinically aggressive manner)[59, 68] and are genetically different from infiltrative tumors, even though most patients continue to be treated similarly to those with conventional PTC[69]. So, in 2016 Nikiforov et al.,[69] proposed that noninvasive EFVPTC should be termed as "noninvasive follicular thyroid neoplasms with papillary-like nuclear features" (NIFTP). This study also suggests that the clinical management of these patients can be deescalated because they are unlikely to benefit from completion thyroidectomy and radioactive iodine therapy. This reclassification intends to affect a large population of patients worldwide to achieve a significant reduction in psychological and clinical consequences associated with the diagnosis of cancer[68].

Besides the cPTC and the FVPTC there are several other histological subtypes of PTC (Table1) with lower incidences that present, the characteristic papillary-like nuclei, and specific growth patterns, cell types, stromal changes and prognosis[61].

Table1. Variants of PTC

Classic*
Follicular*
Macrofollicular*
Oncocytic*
Clear cell*
Diffuse sclerosing*
Tall cell*
Columnar cell*
Solid*
Cribriform morular*
Papillary carcinoma with fasciitis-like stroma*
Papillary carcinoma with focal insular component*
Papillary carcinoma with squamous cell or mucoepidermoid carcinoma*
Papillary carcinoma with spindle and giant cell carcinoma*
Papillary microcarcinoma*
PTC with prominent hobnail features#

*World Health Organtzation[61], #Asioli et al.,2010[70]

PTC tend to be a biological indolent tumor, and PTC patients have a good prognosis (10 year survival is >90%), however the presence of vascular invasion and nuclear atypia may be adverse prognostic signs[61]. The World Health Organization (WHO) recognizes some variants: the tall-cell variant and the columnar cell variant as a subcategory of biological aggressive variants[61]. PTC with prominent hobnail features was also described as an aggressive variant compared do classic PTC[70].

FTC represents 10-15% of all TC. FTCs are invasive neoplasms of follicular origin that lack the typical nuclear features of PTC[61]. They can be divided in two major groups: minimally invasive (invasion limited to the capsule and/or vascular invasion) and widely invasive (widespread infiltration of adjacent thyroid tissues and/or blood vessels). The identification of capsular or vascular invasion differentiates FTC from follicular adenoma (FTA); carcinomas tend to have ticker and more irregular capsule[61, 71]. Patients with minimally invasive FTCs have a very

low long term mortality (3-5%), while widely invasive FTC patients have a long term mortality up to 50%[61].

FTC tends to be more aggressive compared to PTC. FCT rarely metastasizes to regional lymph nodes (5%), instead they usually present blood vessel invasion and ~20% of them present distant metastases, more frequently found in lung and bone[61, 71]. FTC encompass two variants: oncocytic variant (more likely to recur and cause death by local invasion compared to conventional FTC) and clear cell variant[61].

2.5 Prognostic biomarkers

In general, DTC has a favorable prognosis, but it is of great importance to identify at the time of diagnosis those patients who have a high risk of progressive disease and DTC-related death. The importance of recognizing prognostic variables is relevant for the optimal management of DTC, e.g. the extent of thyroid surgery and the indications for postoperative radioiodine therapy. A recognized prognostic classification is also critical for the comparison of treatment results[72].

2.5.1 Age

Age at diagnosis is a critical predictor of patient outcome. TC in children tend to have a low mortality rate, even with extensive disease or distant metastases at presentation[73], the best indicator of outcome in this group is response to radioactive iodide (RAI) therapy[74]. Older patients tend to have aggressive histological variants, extensive neck and distant metastases at diagnosis, tumors tend to be more undifferentiated and metastases uptake less ^{131}I compared to younger patients[75].

The prognostic cut off value for thyroid cancer considered by the Union for International Cancer Control/American Joint Committee on Cancer (UICC/AJCC) until the 7th edition to the calculation of the TNM (Tumor, Node, Metastases) staging (that will be further addressed) for age was 45 years, with patients with ≥45 years presenting worse prognosis compared to patients with <45 years[76] (Table 2).

2.5.2 Gender

Even though the incidence of DTC is higher in females compared to males (discussed above), the mortality rates are higher among males than women[77]. Rates of extrathyroidal extension, likelihood of lymph node metastases and recurrence are higher in males compared to women (from 1988 to 2007)[42].

2.5.3 Tumor size

Tumor size correlates with patients' outcome. Larger tumors are associated with higher recurrence rates and worse prognosis[72, 78, 79]. Larger tumors present more often locoregional and distant metastases, and the risk of recurrent disease and cancer specific mortality increases linearly with tumor size[77].

2.5.4 Extrathyroidal extension

The presence of extrathyroidal extension (ET) is also a prognostic factor in TC, its presence increases the risk of recurrence[72, 78, 79]. There are different degrees of ET, according to the 7th edition of AJCC/UICC staging system: ET could be divided in minimal (being classified as T3), and gross (being classified as T4)[76] (Table 2). Studies are concordant about the association of gross ET and higher risk of recurrence, however, the impact of minimal ET on recurrence rate and prognosis is controversial, some studies demonstrated that minimal ET had no risk/prognostic impact[80-82]

2.5.5 Lymph node metastases

Lymph node metastases, *per se*, have no prognostic impact. Several studies have found no difference in survival between patients with or without lymph node metastases[78, 79, 83]. Only one study demonstrated that the presence of lymph node metastases may have impact on survival, but only according to patients' age: in patients with <45 years, lymph node metastases had no impact on survival, while in patients with ≥45years it was associated with increased risk of death[84].

2.5.6 Distant metastases

The presence of distant metastases in DTC is rare at the time of diagnosis and even rarer after the initial treatment with RAI (2.5-5%)[85] but very relevant. Mortality is higher in patients with distant disease, with a 50% survival at 3.5 years[86]. Nonetheless, even in the presence of distant metastases there are some aspects that can affect significantly the patients' outcome: patients presenting distant metastases initially, appear to have relatively favorable outcomes compared with DTC patients who developed distant metastases after initial treatment[87], furthermore, survival is significantly improved in those cases which distant metastases remain avid to ^{131}I therapy[85, 86] . In DTC, the ability to uptake ^{131}I has a great impact on survival, even in the presence of distant metastases, so the therapy response is a prognostic factor of great value[85], this issue will be further discussed in the "treatment" section.

2.5.7 Tumor staging systems

In order to separate patients with low risk of recurrence or death, from those with intermediate to high risk, some staging/grading systems were developed using different combinations of prognostic factors. Most of the prognostic factors used to calculate risk can be assessed at the time of diagnosis. Staging systems are used to select the most appropriated initial treatment[88].

The European Thyroid Association (ETA) and the American Thyroid Association (ATA) recommend the use of Tumor, Node, Metastasis (TNM) classification of the UICC/AJCC for DTC staging, represented in Table 2.

When we performed our studies, the current edition was the 7th, so every time we refer to TNM staging we will be using 7th edition criteria (Table 2). Recently, some studies recommended a change in the cutoff age from 45 years to 55 years, defending that this change would prevent over-staging in low-risk patients and prevent over- treatment[89, 90]. Consequently, in the 8th edition of UICC/AJCC staging system for DTC changed the cut off value from 45 years to 55 years[91]. Moreover, in the 8thedition, the definition of T3 has been revised and a new T category emerged: T3a- tumor more than 4 cm in greatest dimension, limited to the thyroid; T3b- tumor of any size

with gross extrathyroidal extension invading strap muscles (sternothyroid, or omohyoid muscles)[91]. There is a representation of the 8th edition of AJCC/UICC staging system for DTC in Appendix II.

UICC/AJCC staging system for differentiated thyroid carcinoma

Adapted from UICC/AJCC TNM 7th edition #

T- Primary Tumor

T1- Tumor ≤2cm in greatest dimension limited to the thyroid

T1a- Tumor <1cm, limited to the thyroid

T1b-Tumor >1cm but ≤2cm in greatest dimension limited to the thyroid

T2- Tumor >2cm but ≤4cm in greatest dimension limited to the thyroid

T3-Tumour > 4 cm in greatest dimension, limited to the thyroid or any tumor with minimal extrathyroidal extension (e.g., extension to sternohyoid muscle or perithyroid soft tissues)

T4a*-Tumor of any size extending beyond the thyroid capsule to invade subcutaneous soft tissues, larynx, trachea, esophagus or recurrent laryngeal nerve

T4b*-Tumor invade prevertebral fascia or encases carotid artery or mediastinal vessels

N- Regional lymph nodes

Nx- Regional lymph nodes cannot be assessed

N0- No regional lymph node metastases

N1a- Metastases to Level VI (pretracheal, paratracheal, and prelaryngeal/delphian lymph nodes)

N1b- Metastases to unilateral, bilateral, or contralateral cervical (Levels I, II, III, IV, or V) or retropharyngeal or superior mediastinal lymph nodes (Level VII)

M- Distant metastases

Mx- Distant metastases cannot be assessed

M0- No distant metastases

M1- Presence of distant metastases

Staging

Stage<45 years old		**Stage ≥45 years old**	
Stage I		Stage I	
	Any T. Any N. M0		T1. N0. M0
Stage II		Stage II	
	Any T. Any N. M1		T2. N0. M0
		Stage III	
			T3. N0. M0
			T1. N1a.M0
			T2. N1a.M0
			T3. N1a.M0
		Stage IVa	
			T4a. N0. M0
			T4a. N1a. M0
			T1. N1b.M0
			T2. N1b.M0
			T3. N1b.M0
			T4a.N1b.M0
		Stage IVb	
			T4b. Any N. M0
		Stage IVc	
			Any T. Any N. M1

*All anaplastic thyroid carcinoma are considered as T4. # Adapted from AJCC: thyroid. In: Edge SB, Byrd Compton CC, et al. AJCC Cancer staging manual. 7th ed. New York NY: Springer, 2010, 87-96

In addition to TNM staging, other staging systems have been proposed.

Table 3 Summary of other staging systems for thyroid cancer

Staging systems	Prognostic factors involved
AMES (for DTC)[92],	**A**ge, distant **M**etastases, **E**xtent and **S**ize of primary tumor
AGES (for PTC)[93]	**A**ge, tumor **G**rade, **E**xtent and **S**ize
MACIS (for PTC)[83]	Distant **M**etastases, **A**ge, **C**ompleteness of surgery, **I**nvasion of extrathyroidal tissues and **S**ize of the primary tumor
De Groot's clinical classification (for DTC)[94]	Extrathyroidal extension, cervical lymph node metastases, completeness of surgery and distant metastases
NTCTCS (National Thyroid Cancer Treatment Cooperative Study Classification) (for all thyroid carcinomas)[95]	Tumor size, multifocality, extrathyroidal extension, degree of differentiation, cervical lymph node metastases and distant metastases

A comparison of the different prognostic systems in DTC demonstrated that the UICC/AJCC staging system clearly outperforms other prognostic systems[96].

The clinic applicability of these staging classifications for patients with DTC presents certain limitations: they do not contemplate the early diagnosis or recurrence or even therapy sucess[54, 72]. TNM staging was developed to predict the risk of death, since DTC patients present excellent 10 and 20 year disease survival, the most important aspect to predict in DTC patients is the risk of recurrence[54]. As a consequence, ATA (edition of 2009) has created a more functional definition of risk stratification for individual patients that is similar to the one outlined by the ETA[97,98], both proposed a three-tiered clinicopathological risk stratification: low, intermediate and high risk for recurrence. In 2015 ATA[50] guidelines updated some of the categories:

- Low risk patients are those patients that present DTC with no evidence of ET or vascular invasion. Patients with small volume lymph node metastases (clinical N0 or ≤ 5 pathologic N1 micrometastases, <0.2 cm in largest dimension), intrathyroidal encapsulated follicular variant of PTC, intrathyroidal well-differentiated follicular cancer with capsular or minor vascular invasion (<4vessels involved), and intrathyroidal papillary microcarcinomas that are either *BRAF* wild type or *BRAF* mutated;

- Intermediate risk patients demonstrated either microscopic ET, cervical lymph node metastases, RAI avid disease in the neck outside the thyroid bed, vascular invasion or aggressive tumor histology and a subset of patients with lymph node metastases (clinical N1 or >5 pathologic N1 with all involved lymph nodes < 3 cm in largest dimension), intrathyroidal papillary thyroid cancer with a primary tumor of 1-4 cm that is *BRAF* mutated (if known), and multifocal papillary microcarcinoma with extrathyroidal extension and *BRAF* mutated (if known);
- Finally, high risk patients have gross ET, incomplete tumor resection, distant metastases, or inappropriate post-operative serum Tg values. Patients with large volume lymph node involvement (any metastatic lymph node ≥ 3 cm in largest dimension), and FTC with extensive vascular invasion (> 4 foci of vascular invasion or extracapsular vascular invasion)[50].

2.6 Genetic predictors

Several genetic alterations have been identified in TC, and a putative role as molecular biomarkers of aggressiveness has been assigned for some of them. The importance of genetic markers for predicting thyroid cancer outcome is limited by the prominence of clinical, histopathological, and other context-driven features. Clinical and histopathological prognostic factors remain much more important than genetic factors for diagnostic and prognostic purposes[88]. This conclusion is, however, challenged almost every day by the publication of new molecular data in the different types of TC. The most important of such publications was the "Integrated genomic characterization of papillary thyroid carcinoma" that provided a detailed description of the genomic landscape of 496 cases of PTC under the auspices of The Cancer Genome Atlas (TCGA) Research Network Initiative[99]. This study highlighted the importance of the genetic background by demonstrating that PTCs can be grouped according to their genetic background, with each group harboring distinct characteristics, concerning for example differentiation[99].

The genetic alterations that are more prevalent and/or seem to play a more important prognostic role in DTC are: *RAS*, *BRAF*, and *TERT* promoter (*TERT*p) mutations[100].

2.6.1 *RAS* mutations

RAS are small GTPase-proteins that act as a molecular switch propagating signals from tyrosine kinase and non-tyrosine kinase receptors and activating the Mitogen Activated Protein Kinases (MAPK) and other signalling pathways. *RAS* mutations are more prevalent and seem to be more relevant as a prognostic indicator in follicular patterned lesions (FVPTC, and FTC) than in classic PTC[101]. *RAS* mutations are less prevalent in PTC (10%) than in FTC (25-30%)[102, 103], and within PTC *RAS* mutations are rare in the classic form of PTC whereas, in FVPTC, its prevalence falls within the range of other follicular patterned tumors (~25%)[104].

All the three *RAS* genes (H, K, N-*RAS*) were shown to be mutated in both benign and malignant thyroid tumours, which brings some controversy on the prognostic value of RAS mutations in thyroid cancer. Specifically, in DTC, studies reported an association between (N) *RAS* mutation and distant metastases and/or lower survival in FTC[105, 106].

RAS mutations are present in DTC with areas of dedifferentiation, furthermore their prevalence is greater in PDTC and ATC than in DTC[107] which may indicate that they canpredispose to differentiation loss in TC. However, a recent study with a high number of PTCs demonstrated that *RAS*-like PTCs are more differentiated, as least compared to *BRAF*V600E driven PTCS[99].

It has been difficult to demonstrate the prognostic value of *RAS* mutations due to the relatively small size of the majority of the series (in particular concerning FTCs, which are less frequent than PTCs) and the too short follow-up in most situations. Large, multicentric studies will be necessary to establish definitely the prognostic value of *RAS* mutations.

2.6.2 *BRAF* mutation

BRAF gene encodes a serine/threonine kinase that belongs to RAS-RAF-MEK-ERK- MAP Kinase pathway, whose biological role is to mediate cellular responses to growth factors. There are several *BRAF* mutations, being *BRAF*T1796A (in exon 15), leading to a substitution of a

valine by a glutamic acid at position 600 (V600E), largely more prevalent. Such mutation causes an increased *BRAF* kinase activity and subsequent phosphorylation of MEK1/2 and ERK1/2, turning the activation of the MAP kinase pathway independent from upstream factors activation[108].

*BRAF*V600E mutation is the most prevalent point mutation in PTC being present in 36-69% of cases[101]. It rarely co-exists with other prevalent genetic events such as *RET/PTC* rearrangement[109] or *RAS* mutation[110]. *BRAF*V600E mutation exhibits a strong genotype-phenotype association; it is (almost) exclusively detected in PTC exhibiting papillary or mixed follicular/papillary growth pattern[111].

Although functional studies, using thyroid-targeted *BRAF*V600E transgenic mice[112] and *BRAF*V600E transfected thyroid cell lines[113], indicate that *BRAF* mutations lead to an "aggressive type" of PTC, several other studies, addressing the correlation between *BRAF*V600E and the clinical features of PTC provided discrepant results (see below).

Some studies reported significant associations between *BRAF* mutation and poor prognostic indicators like older age[114, 115], male gender[116-118], ET[114, 119], regional metastases[117, 119], distant metastases[120], higher tumour staging[114, 119, 120], tumour size[117, 118, 121] and tumour recurrence[119, 122]. Other studies have not observed the aforementioned associations[123-125].

Recently, a multicenter retrospective study showed that *BRAF*V600E was significantly associated with increased cancer-related mortality among patients with PTC but the association was not independent of several clinicopathological features of aggressiveness[126].

We observed that *BRAF*V600E PTCs tended to occur in older patients and did not exhibit a significant association with signs of clinicopathological aggressiveness, like larger size, ET, vascular invasion and lymph node metastases[111, 127], or poor circumscription[127]. This does not mean, however, that *BRAF* mutation cannot contribute for progression of PTCs towards less differentiated carcinomas in the appropriate context, since our group and others[55, 114, 120] detected *BRAF*V600E mutation in 10-35% of ATC. A more recent study demonstrated that *BRAF*V600E driven PTCs are less differentiated compare to *RAS* driven PTCs[99].

In fact, it is widely demonstrated that *BRAF*V600E is associated with a decrease of several "thyroid specific genes" or "iodine handling genes"[122, 128, 129], this issue will be further discussed in "NIS expression regulation in thyroid cancer" section.

2.6.3 *TERT* promoter mutations

Recently, mutations in the promoter region of telomerase (*TERT*) gene were reported in follicular cell-derived thyroid carcinomas (FCDTC)[130-132]. These mutations occur in two hotspot positions, located -124bp and -146bp upstream from the ATG start site (-124G>A and -146G>A, C>T on opposite strand) and confer enhanced *TERT* promoter activity, putatively by generating a consensus binding site (GGAA) for ETS transcription factors within the *TERT* promoter region[133].

In a large series of 469 carcinomas, our group found *TERT* promoter mutations (*TERTp*) in 7.5 % of PTC and 17.1 % of FTC[134]. The majority (about 80%) of mutated cases present the -124G>A mutation.

In DTC, *TERT*p mutations were associated with older age, larger tumours and presence of distant mestastases[134, 135]. Furthermore, patients harbouring *TERT*p mutations were less prone to be disease-free at the end of follow-up. Similar results were found in three other studies[136-138]. Patients with *TERT*-mutated tumours were submitted to more treatments with radioiodine with higher cumulative doses, as well as to other treatment modalities like surgery for recurrent disease, external beam irradiation or treatment with tyrosine kinase inhibitors (TKI)[134]. Furthermore, patients with tumours harbouring *TERT* promoter mutations had increased disease-specific mortality, and this finding was independent of age and gender[134].

In PTC, *TERT*p mutations were significantly more frequent in *BRAF* mutated tumours[132, 134]. *TERT*p mutations were associated with increased mRNA expression, and this increase was more pronounced in tumours harbouring both *BRAF* and *TERT* promoter mutations[130].

Since *BRAF* has also been associated with worse prognosis in some studies, several authors hypothesized that both mutations could cooperate towards a worse prognosis[132, 139]. Multicentric

studies with large series of patients will be necessary to clarify if the addition of *BRAF* mutational status to a *TERT*-mutated tumor has indeed value for prognostic stratification[140].

The prognostic biomarkers, staging systems and genetic predictors are very useful in the estimation of the risk and stratification of TC patients for different treatment approaches.

2.7 Treatment of differentiated thyroid carcinoma

The main goals of DTC treatment are: remove completely the primary tumor and lymph node metastases (when present); minimize the risk of disease recurrence and metastatic spread; permit accurate staging and risk stratification of disease; favor accurate long-term surveillance for disease recurrence and minimize treatment related morbidity[50].

The initial treatment for DTC is total or near-total thyroidectomy whenever the diagnosis is made before surgery and the nodule is ≥1 cm, or regardless of the size if there is metastatic, multifocal or familial DTC[98].

After initial surgery, the second pillar of treatment for DTC is RAI therapy in order to eliminate thyroid and tumor remnants and/or metastases[50, 141, 142].

Patients are designated for RAI treatment or not, according to a combination of some postoperative findings. RAI treatment is: not considered if tumor size ≤1cm T1a, uni or multifocal; not routinely considered if tumor size >1cm ≤4cm (T1b-T2) or follicular cancer with no or minimal (<4foci) vascular invasion; considered if tumor size ≥3cm (T3) (7^{th} edition UICC/AJCC TNM staging), presence of microscopic ET (T3), presence of lymph node metastases in central compartment (N1a) or lateral neck lymph node metastases (N1b); absolutely considered if follicular thyroid cancer with extensive vascular invasion (>4foci), gross extrathyroidal extension (T4) or presence of distant metastases[50].

Due to their well-differentiated nature, DTC cells often retain some degree of differentiation (in comparison to normal thyroid), that includes: NIS expression and functionality and the ability to uptake iodine, the production and secretion of Tg and expression of TSHR on their surfaces[143,144-146]. The administration of RAI refers to the administration of the radioactive isotope ^{131}I, that due to NIS expression and preserved functionality, is uptaken by thyroid

normal/tumors cells[147]. Once in the interior of the cells, ^{131}I decays and emits beta radiation with a mean tissue penetration of 1mm, as well as a more deeply penetrating gamma radiation that can be detected by scintigraphy. When RAI is administered, the tumor receives a high radiation dose (causing cell death), while the surrounding tissue is largely spared. Because of this biological property of thyroid tumor cells (preserved NIS expression and functionality) radiotherapy can be delivered specifically to the tumor tissue[141]. In order to increase NIS expression/functionality, prior to ^{131}I administration, patients are subjected to an increase in TSH levels in the serum (≥30mU/L), either by administration of recombinant TSH or discontinuing treatment with levothyroxine for 4-5 weeks[50, 147].

RAI therapy is a very efficient targeted radiotherapy; it is given on an adjuvant basis after thyroidectomy, to destroy thyroid normal/tumor remnants and/or distant metastases, decreasing the risk of locoregional recurrences. Moreover, it also increases the sensitivity and specificity of follow-up testing for DTC persistence or recurrence:

- measurements of serum Tg as a tumor marker (detectable serum Tg levels indicate persistence/recurrent of disease), and
- diagnostic radioiodine whole body scintigraphy (detection of RAI uptake indicates persistent/recurrent disease[142,147].

Unfortunately, a subgroup of DTC patient (4-23%) develop distant metastases, worsening their prognosis[148]; patients are at increased risk to succumb to the disease when the tumor loses the ability to uptake RAI, which occurs in approximately 26-60% of the patients with recurrent disease[149]. The loss of NIS expression/function is thought to be the major molecular mechanism that contributes to RAI refractoriness[150]. This subgroup of DTC patients represents a real challenge in TC field because, although all clinicopathological prognostic factors, genetic predictors, and staging systems, it is still not possible to predict which DTCs patients that will course with high morbidity and eventually mortality at initial diagnosis.

RAI treatments are highly effective in younger patients and with small metastases[85]. A study compared the survival rates between patients with recurrences with and without ^{131}I uptake:

the 10 year survival rate was of 92% in patients with ^{131}I uptake compared to 10% in those patients without any initial ^{131}I uptake[85]. Another study addressed the prognostic factors of DTC patients with lung metastases, and the ^{131}I non avidity, observed more often in late metastases, was the only independent predictive factor of poor prognosis[148]. In conclusion, the response of DTC remnants/recurrences to RAI is a critical prognostic factor.

Having this in mind, ATA 2015 guidelines proposed a system to estimate the risk of recurrence, during follow-up, based on RAI response[50].

Table 4.ATA risk assessment during follow-up

Excellent response (1-4% recurrence)
. Imaging negative for disease recurrence
. Serum thyroglobulin concentration lower than 0.2ng/ml basal or higher than 1ng/ml TSH stimulated
Indeterminate response (15-20% recurrence)
. Non-specific findings on imaging studies
. Serum thyroglobulin concentration 0.2-1ng/ml basal or TSH stimulated or thyroglobulin antibodies stable or decreasing
Biochemical incomplete response (20% recurrence)
. Imaging negative for disease recurrence
. Serum thyroglobulin concentration higher than 1ng/ml basal or higher than 10ng/ml TSH stimulated, or increasing thyroglobulin antibodies concentrations
Structural incomplete response (50-85% recurrence)
. Structural (neck ultrasound, CT or MRI) or functional (whole body scan 18F-fluorodeoxyglucose PET) evidence of disease in imaging studies

CT computed tomography. MRI Magnetic resonance imaging

According to ATA guidelines 2015, a radioiodine-refractory structurally-evident DTC is defined in four basic ways (under similar conditions of TSH stimulation and low iodine intake):

1) the malignant/metastatic tissue does not ever concentrate radioiodine (no uptake outside the thyroid bed at the first diagnostic or therapeutic WBS);

2) the tumor tissue loses the ability to concentrate radioiodine after previous evidence of RAI-avid disease (in the absence of stable iodine contamination);

3) radioiodine is concentrated in some lesions but not in others;

4) metastatic disease progresses despite significant concentration of radioiodine[50].

When a patient with DTC is classified as refractory to radioiodine, there is no indication for further radioiodine treatment[50]. Management of RAIR DTC is a real challenge, that vary from active surveillance (only biochemical evidence of disease and/or small cervical lymph node metastases and/or stable or slowly growing distant metastases) to localized therapy (when there is only one metastatic site and/or there are only a few progressive lesions) being the surgery the best therapeutic option, to systemic therapy (rapidly progressive disease and/or larger tumor burden and/or symptomatic)[150].

Systemic therapies consisted more often in TKI and their goal is to stop progression[150]. Sorafenib and lenvatinib are both TKI approved by Food and Drug Administration (FDA) to treat RAI refractory DTCs (in 2013 and 2015, respectively). In some cases, sorafenib caused lesion shrinkage[151].Unfortunately neither sorafenib[152] or lenvatinib[153] show any improvement in patient overall survival, but those treatments improved progression-free survival rates when compared to placebo[152, 153].

Even though TKIs may not seem very promising in controlling the development of advanced refractory DTC, treatment of RAIR patients with selumetinib was able to enhance RAI uptake and even resssensitize some tumors to RAI therapy[154]. As far as our knowledge, this study was not continued, but it calls our attention to the importance of developing new treatments focused not only in stop tumor progression, but specially in restoring NIS expression/function.

3. mTOR pathway

Mammalian target of rapamycin (mTOR) is a multidomain Ser/Thr kinase that belongs to the phosphoinositide 3-kinase (PI3K) pathway. mTOR pathway can be activated by diverse exogenous stimuli, such as growth factors, nutrients, energy and stress signals and essential signaling pathways: the canonical pathway of mTOR activation depends on mitogen-driven signaling through PI3K/AKT, although alternative non-AKT dependent activation through the RAS/MEK/ERK pathway is now recognized[155].

mTOR activity in the cell is carried out by two distinct complexes: mTORC1 complex made up by mTOR, raptor, mLST8, Deptor and PRAS40) and mTORC2 complex (composed by mTOR, rictor, mSin1, Deptor, Protor, HSp70and mLST8). Both complexes are activated by different stimuli and have different physiological functions[155-158]. When mTOR is phosphorylated at Ser2448 it can be part of any mTOR complexes, whereas phosphorylation in Ser2841 it is mTORC1 unique[159].

mTORC1 signaling has been more studied and is better understood compared to mTORC2 signaling[156]. mTORC1 is particularly sensitive to acute treatment with rapamycin, during some years mTORC2 complex was considered insensitive to rapamycin treatment, but recent evidences demonstrated that mTORC2 complex is also inhibited by rapamycin following chronic exposure[160].

mTORC1 complex is activated by the presence of growth factors and hormones such as insulin[161], nutrients such as amino-acids[162] and cytokines such as tumor necrosis factor α- TNF α[163]. mTORC1 is active when cells are at a high energy state[164] and in the presence of oxigen[165, 166], being inhibited in response to low ATP levels and hypoxia [164-166] .

Regardless of the activating source, mTORC1 will phosphorylate its downstream effectors: the serine/threonine kinase S6K1 and S6K2, that in its turn will phosphorylate S6 (40S ribossomal protein S6)[167, 168], and the eukaryotic initiation factor E binding protein (4EBP1)[169] (Figure 3). pS6 will enhance mRNA translation particularly of ribossomal protein, elongation factors and insulin growth factor 2[155]. Phosphorylation of 4EBP1 promotes the dissociation of 4EBP1/eIF4E

(eukaryotic translation initiation factor 4E). Once free eIF4E will form a complex with other proteins culminating in an increased translation of its target genes, including cyclin D1, which are required to cell cycle progression (from G1 to S phase)[155] (Figure 3). Furthermore, mTORC1 also stimulates adipogenesis[170] and blocks autophagy[171]. Summing up, mTORC1 controls cell growth, proliferation, metabolism and survival[156].

pS6 is phosphorylated by S6K1/2 at the C-terminus on Ser236, Ser235, Ser240, Ser244 and Ser247[172]. In addition to being phosphorylated by S6K1/2 in a mTOR dependent way, some evidences demonstrated that S6 can also be phosphorylated independently of mTORC1 activity. S6K1-/-/2-/- knock-out mice, were found to display no phosphorylation of pS6 at Ser240/244, but persistent phosphorylation at Ser235/236, revealing the presence of another *in vivo* pS6 kinase, that was identified as p90 ribosomal S6 kinase (RSK)[173]. RSK can phosphorylate S6 in response to RAS/ERK pathway, serum and growth factors independently of mTORC1[168]. Furthermore, another kinase, the casein kinase 1 (CK1) was also described as being able to phosphorylate pS6 (Ser247) also independently of mTORC1. It is important to refer that RAS/ERK pathway can contribute to phosphorylation of S6 also in a mTOR dependent way, briefly, ERK and RSK promotes TSC1/TSC2 complex dissociation, which drives the small GTPase Rheb into active state, leading to mTORC1 activation at Ser2448[155].

mTORC2 complex is not responsive to nutrient stimulation, it respond to growth factors via PI3K-mediated mechanism[174]. Besides that, mTORC2 is also regulated by mTORC1; mTORC1 can negatively influence mTORC2 function via S6K1 phosphorylation of rictor and Sin1[175-177].

Once activated, mTORC2 phosphorylates AKT at Ser473, protein kinase C α (PKC α), glucocorticoid-induced protein kinase (SGK1) and paxilin[178-182] and can also affect the activity of Rho GTPases[183](Figure 3). Recently, a study demonstrated that mTORC2 can phosphorylate insulin receptor and insulin growth factor receptor[184]. According to its downstream effectors, mTORC2 can contribute to actin cytoskeleton remodeling and cell migration, through Rho GTPases, paxilin[183] and PKC α [185](Figure 3). Additionally, mTORC2 can influence cell survival,

growth, migration and proliferation through its effects on AKT, SGK1, (Figure 3) insulin receptor and insulin like growth factor receptor[179, 180, 184, 185].

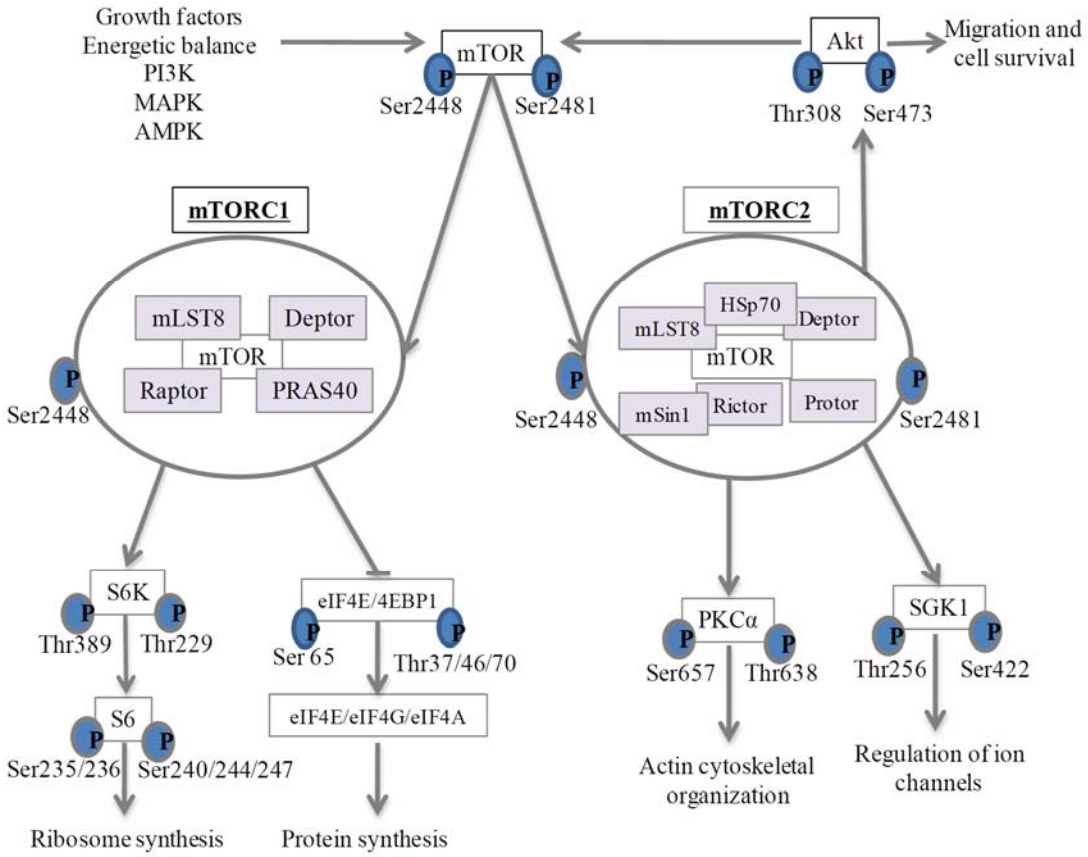

Figure 3. Representative diagram of mTORC1 and mTORC2 assembly and respective main downstream effectors.

3.1 mTOR pathway in cancer: different roles of mTORC1 and mTORC2 complexes

Taking into consideration the functions of the mTOR pathway in cell growth and metabolism, it is not surprising that this pathways is overactivated in a large variety of human neoplasms being in some models implicated in tumor growth, metastases and/or correlated with worse prognosis[155].

Aberrant mTOR pathway activation in cancer can be a consequence of oncogene stimulation or loss of tumor supressors[186]. Mutations in mTOR, PI3K, mutations/amplifications

of AKT and downregulation of PTEN (mTOR pathway blocker) are genetic events that contribute to mTOR pathway overactivation in cancer[155].

The contribution of the mTORC1 complex to tumor progression is better understood in comparison to mTORC2. mTORC1 (through its downstream effectors pS6 and p4EBP1) potentiate the severity of tumor progression through numerous molecular mechanisms like tumor proliferation, growth and resistance to apoptosis[187]. Overexpression of the mTORC1 downstream effector pS6 has been implicated in tumor progression and/or worse prognosis in melanoma, ovarian, lung, gastric cancer and esophageal squamous cell carcinoma[188-192], on the other hand 4EBP1 expression has been associated with progression and worse prognosis in renal cell carcinoma, breast and lung cancer[193-195].

mTORC2 complex also seems to play a role in cancer. pAKT Ser473 expression was associated with invasion in bladder cancer[182] and with metastization in breast and gastric cancer[196, 197]. Furthermore, mTORC2 complex has been associated with an increase in cell migration in models of renal cell carcinoma, breast cancer and gliomas[198-200].

Even though there are evidences that both mTORC1 and C2 complexes are involved with invasion and metastization[201, 202], mTORC2 complex is more often associated with these features[182, 196, 197, 199, 203], when compared to mTORC1 complex[198]. In fact activation of AKT on Ser473 is associated with essential steps of metastization, such as the loss of expression of the adhesion molecule E-cadherin, thereby permitting cell detachment[204].

3.2 mTOR inhibitors

Rapamycin was the first identified mTOR inhibitor and nowadays several analogs are also available, known as rapalogs[155]. They all share the same mechanism of action, but new rapalogs tend to be more soluble and stable compared to rapamycin[156].

Rapamycin/rapalogs form a complex with FKBP12-binding protein (FKBP12) that will bind to the FKBP12/rapamycin-binding (FRB) domain of mTOR only when mTOR is associated with other components of mTORC1. The FKBP12/rapamycin complex results in the dissociation

of Raptor from mTORC1 and loss of contact between mTORC1 and its substrates[205]. As referred, it was demonstrated that chronic treatment with rapamycin can also disrupt mTORC2 complex[160].

Some rapalogs are currently used in cancer therapy and others are in clinical trial, for example RAD001/everolimus was approved by FDA for the treatment of advanced renal cell carcinoma, subependymal giant cell astrocytoma, non resectable neuroendocrine pancreatic tumors and advanced estrogen receptor positive/HER2 negative breast carcinoma (in association with other drugs)[205].

However, the efficacy of rapamycin/rapalogs as broad-based monotherapy for the treatment of cancer has not been as promising as initially expected, being limited to a subset of solid tumors[156, 205]. One possible explanation is the failure of acute inhibition of the mTORC2 complex[155, 156]. So, a new generation of mTOR inhibitors as emerged, those drugs bind to the ATP binding site of mTOR decreasing its kinase activity, known as mTORC1/mTORC2 dual inhibitors[156, 205]. Examples of dual mTOR inhibitors are AZD8055, AZD2014, OSI-027, INK128/ MLN-0128 and Torin2 among others[206], some with promising results in preclinical evaluations[207].

3.3 mTOR pathway in thyroid carcinoma

Both mTORC1 and C2 complexes are overactivated in PTCs: higher expression of their downstream effectors (pS6, p4EBP1 and pAKT) has been identified in PTCs compared to normal adjacent tissues[208-210]. In medullary thyroid carcinoma (MTC), mTOR pathway is also activated, pS6 expression is more intense in *RAS* mutated MTC, associated with the presence of lymph node metastases and significantly increased in invasive tumors [211].

Despite being activated, the impact of mTOR pathway in DTC clinical behavior remains largely unknown. There is some dispersed evidence that pS6, p4EBP1, pAKT and/or Sin 1 expression is higher in PTC with more aggressive histology compared to classical PTC[212, 213], and that cPTC harboring *BRAF*V600E mutation expressed higher levels of pmTOR, pS6 and pAKT[210] compared to *BRAF*WT, presenting though, an overactivation of components of both mTORC1 and C2 complexes. Finally, it was reported that pAKT was predominantly found in regions of capsular invasion, and assumed a nuclear translocation in the invasive regions of PTCs[209].

Pharmacologic abrogation of mTOR pathway in TC cell lines by rapamycin caused a decrease on cell viability[210] and treatment with Torin2 lead to a cell cycle arrest and increased apoptosis[58]. Torin2 also impaired tumor growth *in vivo*[214]. These results demonstrate that mTOR pathway is involved in cancer cell proliferation and survival. Inhibition of mTORC1 by rapamycin in thyrocytes and thyroid cancer cell lines also caused an incresase of NIS expression and RAI uptake, this particularity was previously adressed in "NIS expression regulation in thyroid carcinoma" section.

Besides being implicated in proliferation and survival, mTOR pathway, and in particular the mTORC2 complex, seems to play an important role in metastization, Studies in a murine model of thyroid cancer (that spontaneously develops distant metastases) demonstrated that PI3K had a preponderant role in metastization[215], and that effect could be mediated by AKT, since it was shown, that AKT deficiency delayed tumor progression, vascular invasion and distant metastases[216]. Finally, it was also demonstrated that activated AKT (pAKT Ser473) nuclear distribution may be relevant to both initiation and sustaining of metastases[217].

Summarizing, it seems that the mTOR pathway may play important roles in thyroid cancer, from cell proliferation, resistance to apoptosis, metastization, and potentially in RAI therapy resistance, becoming a very interesting therapeutic target. The elucidation of the role of mTOR complexes in TC is important since it was recently reported that everolimus demonstrated a clinically relevant antitumor activity in patients with advanced DTC[218].

So, it is very important to understand the impact of the mTOR pathway on TC behavior and therapy resistance, in order to find the best therapeutic strategy to overcome metastatic radioiodine refractory DTCs.

4. NIS expression in thyroid carcinoma

Since NIS appears as a central molecule for RAI therapy success, many studies addressed its expression and/or expression regulation in TC. The following section will address the state of the art concerning NIS expression in thyroid tumors and what are/could be the molecular mechanisms that affect its expression/function.

Many studies, in thyroid cell lines as well as in animal models of TC and in samples of human TC have been performed, in order to understand the molecular mechanisms that lead to NIS downregulation. NIS expression has been addressed by different methodologies, namely quantitative real-time (qRT-PCR) and/or immunohistochemistry (IHC). qRT-PCR is more sensitive compared to IHC, nonetheless *SLC5A5* mRNA levels may not predict the final amount of functional NIS molecule. *SLC5A5* mRNA is further processed and converted to protein, and then subjected to post transcriptional events that can affect its location and function[219], which is very important since NIS is only functional when targeted into the plasma membrane.

4.1 *SLC5A5* mRNA expression in thyroid carcinoma

The vast majority of the studies addressing *SLC5A5* mRNA levels are concordant; *SLC5A5* mRNA expression is decreased in TC compared to normal adjacent thyroid[220-223]. As far as we are aware, only one study reported an increased *SLC5A5* mRNA expression in PTCs compared to normal adjacent tissues[224]. Nevertheless, *SLC5A5* mRNA levels seem to be of limited value in predicting NIS protein expression and function.

Lower mRNA levels in general lead to reduced protein levels, but the opposite may not be true, a positive or high mRNA expression does not always correspond to higher protein levels or to functionality. Indeed, previous studies reported positive *SLC5A5* mRNA levels in lymph node metastases of DTC that did not uptake RAI [219] and it was observed similar *SLC5A5* mRNA levels in recurrences of thyroid cancer with and without ^{131}I uptake ability[223]. These observations lead us to believe that other mechanisms, other than genetic control over NIS transcription (like post transcriptional events) might be involved in the failure of RAI therapy.

To add an additional level of complexity we must be aware that *SLC5A5* mRNA levels in primary tumors might not be indicative of the *SLC5A5* expression in metastatic tissues[225]: Park et al.,2000[226] addressed *SLC5A5* mRNA expression in primary tumors and respective lymph node metastases and observed that *SLC5A5* mRNA expression in lymph node metastases was lower compared to corresponding primary tumors[226]. Another study compared *SLC5A5* mRNA expression in primary DTCs with the ability of the correspondent metastases to uptake ^{131}I: what was observed was that metastases from primary DTCs with or without *SLC5A5* mRNA expression demonstrated a lower or even absent ^{131}I uptake[227].

It seems that the absence of *SLC5A5* mRNA expression lead to reduced protein levels, but its presence does not guarantee the protein expression/function. Additionally, *SLC5A5* mRNA expression, in primary tumors, does not seem to be indicative of *SLC5A5* mRNA expression or ^{131}I uptake in the corresponding metastases. Nonetheless, in a few studies, lower *SLC5A5* mRNA levels were correlated with larger tumors (≥2cm), early recurrence and/or metastasis[228, 229], indicating that a lower *SLC5A5* mRNA expression in primary tumors might be associated with higher tumor aggressiveness.

4.2 NIS protein expression in thyroid carcinoma

NIS protein expression (evaluated by IHC) varies through thyroid tissues: in normal thyroid its expression is low and very heterogeneous, only a few follicular cells within some follicles express NIS in the basolateral membrane[230-234], suggesting that in normal conditions, NIS expression is a much regulated process. In Graves' disease (an autoimmune disease in which follicular cells are constantly stimulated to produce thyroid hormones) NIS is widely expressed in membrane across all follicular cells and all follicles. In carcinomas NIS, when present, it is usually expressed in a higher number of cells compared to normal tissue but its expression is mainly cytoplasmic, poorly targeted to the membrane[224, 229, 232-238]. The increased cytoplasmic staining in tumors, even in the presence of low *SLC5A5* mRNA levels[239], has been pointed out as a reason for the decreased RAI uptake in tumors, reflecting a mislocalization of NIS from the membrane, impairing its activity. At variance, other studies question the real significance of NIS

cytoplasmic expression: a study performed with three different monoclonal antibodies raised against NIS suggested that the cytoplasmic NIS expression could be background instead of mislocalized NIS[234]. Since NIS is the molecule for RAI therapy's success, and the mRNA levels may not be very accurate in predicting NIS functionality, some studies addressed the correlation between NIS protein expression on the primary tumor and the RAI uptake of recurrences and/or distant metastases. Just like it was observed for *SLC5A5* mRNA expression, NIS protein expression in the primary tumor did not correspond to NIS protein expression in correspondent lymph node metastases in a significant number of cases[240, 241]. Furthermore, it is unclear if NIS protein expression in primary tumor predicts the ^{131}I uptake by respective recurrences/metastases. Different studies reported that a positive NIS immunoreactivity in primary tumors seemed to be predictive of subsequent positive ^{131}I scans, but a negative NIS staining did not predict ^{131}I negative scans. These studies did not take into account whether NIS was expressed in the membrane or cytoplasm[235, 240] but similar results were verified when membrane expression was considered[241].

Despite the higher number of studies about NIS protein expression, the great majority was performed in small series and did not address possible associations between NIS expression and clinicopathological features and prognosis[129, 224, 230-233, 235-237, 240].

As far as our knowledge, only two studies addressed NIS expression in a significant number of carcinomas (using tissue microarrays (TMA), Morari et al.,2011[229] (265 DTCs) and Wei et al 2013.,(370 PTCs)[238], and found lower NIS protein expression in older patients (>45 years) and in aggressive variants of PTC (compared to conventional PTC).

We think that additional retrospective studies in larger series of DTC are needed, performed in histological sections rather than in TMA, with a more detailed attention to NIS protein location in order to explore the putative impact of NIS protein expression in predicting tumor aggressiveness as well as in prognosis. It is also necessary to compare the analysis of NIS mRNA expression and NIS protein expression, in order to understand what is the best/more informative method to address NIS expression and to understand if NIS protein expression in primary tumor has a predictor value regarding RAI therapy's success.

4.3 NIS expression regulation in thyroid carcinoma

All the "iodine handling genes" (TSHR, NIS, and TPO) are decreased in TC compared to normal tissue, but among them NIS is the one that suffers higher levels of downregulation during the tumor progression[220, 231]. The molecular mechanism that lead to the decrease NIS expression in tumors remains poorly understood, but previous studies demonstrated that mutations in the *SLC5A5* gene are not responsible[242].

Many studies tried to understand the loss of NIS expression as well as its mistargeting to the membrane. In the next section, we will discuss some of the major processes known to impair NIS expression in thyroid cancer.

4.3.1 Genetic background

To understand the mechanisms that contribute to NIS downregulation/ loss of function, some studies addressed the genetic background of recurrent DTCs with and without RAI uptake, and demonstrated that recurrences without uptake showed an enrichment on *BRAF*V600E mutations[223,243].

*BRAF*V600E mutation causes loss of *SLC5A5* mRNA and/or NIS protein expression; these effects are well described in the literatute[85, 122, 129, 229, 244, 245], additionally, this mutation also damages NIS targeting to the membrane[122]. *BRAF*V600E mutation also causes a significant decrease of the majority of the others "iodine handling genes", like TPO and pendrin[128, 246].

Although the correlation between the *BRAF*V600E mutation and the decreased NIS expression is well accepted, pharmacological blockage of MEK (downstream *BRAF* in the MAPKinase pathway) was not able to completely restore NIS expression and RAI uptake, indicating that the *BRAF*V600E impairment of NIS might be, at least in part, MAPK independent[122].

Some studies suggested some possible molecular links between *BRAF*V600E and impaired NIS expression. Riesco-Eizaguirre et al.,[244] demonstrated that *BRAF*V600E induces secretion of functional TGFβ, that in its turn causes NIS downregulation through a TGFβ/Smad signaling mediated process.

Is was also proposed that the *BRAF*V600E mutation is able to reprogram the epigenome of tumor cells by altering not only the histone deacetylation status at critical regions of NIS promoter[247] but also the methylation status of NIS promoter (via upregulation of DNA methyltransferase 1)[248], all this contributing to an impaired *SLC5A5* mRNA expression.

Although the role of *BRAF*V600E mutation on NIS impairment has been widely explored, the role of other frequent mutations in TC such as *RAS* and *TERT*p mutations on NIS expression/function remains largely unknown.

A recent study analyzing a large series of PTC, reported a distinct profile of expression of "iodine handling genes" (being *SLC5A5* one of these genes) between *BRAF*V600E and *RAS*-driven PTCs, with *RAS*-like PTCs having relatively high thyroid differentiation score[99], so it seems that *RAS* mutation may also affect *SLC5A5* expression, although less extensively when compared to *BRAF* mutation. Regarding *TERT*p mutation, there are no studies about its impact on NIS expression but, since *TERT*p mutated DTC patients needed higher number of ^{131}I therapies[134], one may speculate that it also might present impaired NIS expression/function.

In our opinion, the information about the impact of frequent mutations in DTC on NIS expression is scarce and deserves further investigation.

4.3.2 mTOR

Apart from the genetic background, other signaling pathways have been implicated in NIS expression regulation.

PI3K/mTOR pathway is frequently overactivated in TC[210]. In cell cultures of normal thyrocytes from rats and TC cell lines, pharmacological inhibition of PI3K was able to increase *SLC5A5* mRNA expression and radioactive iodine uptake[249]. This observation called the attention to the role of PI3K/mTOR in NIS expression regulation.

Further studies demonstrated that blocking the pathway downstream PI3K, by blocking mTOR with rapamycin (so blocking only mTORC1 complex) was also able to increase or restore *SLC5A5* mRNA expression as well as RAI uptake in normal thyrocytes from rats and thyroid cancer cell lines[250, 251]. Nevertheless, the role of mTORC2 on NIS expression remains unknown.

Taking into consideration all these observations, it seems pertinent to explore the impact of the mTOR pathway on NIS expression and therapy success in human TC, in order to evaluate the potential importance of mTOR inhibitors in refractory DTC. To the best of our knowledge this issue was not previously addressed.

Summing up, NIS expression regulation seems to be modulated by many factors. So, it is not surprising that treatment of TC cell lines with a synergy of drugs targeting the major signaling pathways involved (MAPK, PI3K, mTOR) as well as with epigenetic drugs targeting histone deacetylases (HDACs) inhibitors, have a higher impact on *SLC5A5* mRNA expression and NIS function compared to each drug alone[252]. This synergetic treatment not only increases *SLC5A5* mRNA expression, but also the expression of other iodine handling genes, such as TSHR and TPO[252]. This observation together with the fact that early in tumorigenesis NIS downregulation is accompanied by the loss of other iodine handling genes, lead us to speculate that the loss of NIS expression in TC may not be an isolate event; instead it takes part of the dedifferentiation program that accompanies thyroid tumorigenesis.

Although all this available information, many doubts remain about the role of mTOR status and NIS expression on TC behavior, prognosis and response to therapy.

The understanding of the mTOR pathway impact in TC behavior and in NIS expression is very important because there are already available approved mTOR blockers, which could be very useful if in fact, mTOR pathway emerges as an attractive therapeutic target in for TC.

Regarding NIS expression, even though all the efforts that have been made, it is not clear yet if its expression in the primary tumor is indicative of RAI therapy response. That information is very important, because until know, there is no predictor of RAI therapy response in TC which could be very helpful in the stratification of TC patients and to the development of a more personalized treatment.

Chapter 2. General aims and specific objectives

The majority of DTC patients have a very good prognosis with high rates of cure and/or disease free survival. Despite this there is a small group that eventually will develop recurrences/distant metastases. At present, it is impossible to identify such patients at the time of diagnosis and this limitation may lead to an overtreatment of patients with low risk DTCs. More accurate prognostic biomarkers are necessary for an adequate management of thyroid cancer patients.

mTOR pathway is overactivated in TC but the relative role played bymTORC1 and mTORC2 activation and the prognostic consequences of such activation remain unknown. The significance of NIS expression in the primary tumor for prediction of TC behavior and response to therapy remains also unclarified. mTOR pathway maybe a player in the regulation of NIS expression and function (the central molecule for the success of RAI therapy) becoming a very interesting target to explore to overcome TC therapy resistance. Taking all this into consideration we decided to explore, in this thesis, new prognostic biomarkers by addressing mTOR pathway and NIS expression in thyroid primary tumors, aiming to find some indicators of aggressiveness and therapy resistance.

Our specific objectives were:

1) to explore the role of the mTOR pathway in PTCs through the characterization of the pmTOR Ser2448, pS6 Ser235/236 and pAKT Ser473. In addition to this, to explore possible associations with clinicopathological and molecular features, prognosis and response to therapy,

2) to analyze the role of mTORC1 and mTORC2 complex on *SLC5A5* mRNA expression;

3) to study *SLC5A5* mRNA expression and NIS protein expression and cellular localization in a large series of thyroid primary tumors in an attempt to find associations with clinicopathological and molecular features, prognosis and response to therapy.

Chapter 3. Paper 1. pmTOR is a marker of aggressiveness in papillary thyroid carcinoma

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pmTOR is a marker of aggressiveness in papillary thyroid carcinomas

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Background. *Activation of the mTOR pathway has been observed in thyroid cancer, but the biologic consequences regarding tumor behavior and patient prognosis remain poorly explored.*
Methods. *We aimed to evaluate the associations of the mTOR pathway with clinicopathologic and molecular features and prognosis through the immunocharacterization of pmTOR and pS6 expression (as readouts of the pathway) in a series of 191 papillary thyroid carcinomas.*
Results. *pmTOR expression was associated with distant metastases (*P = *.05) and persistence of disease (*P = *.05). Cases with greater expression of pmTOR were submitted to more* ^{131}*I treatments (r[102] = 0.2;* P = *.02) and a greater cumulative dose of radioactive iodine (r[100] = 0.3;* P = *.01). Positive pmTOR expression showed to be an independent risk factor for distant metastases (odds ratio = 18.2; 95% confidence interval 2.1–157.9;* P = *.01). In contrast, pS6 expression was associated with absence of extrathyroid extension (*P = *.001), well-defined tumor margins (*P = *.05), and wild-type BRAF status (*P = *.01). There was no correlation between the expression of pmTOR and pS6 expression (r[140] = 0.1;* P = *.3).*
Conclusion. *pmTOR expression is an indicator of aggressive, metastatic papillary thyroid carcinoma, being possibly implicated in refractoriness to therapy, while pS6 expression is associated with less aggressive pathologic features. Further studies are needed to understand better the biologic consequences of activation of the mTOR pathway in the behavior of thyroid cancer, namely the contribution of other pmTOR downstream effectors. (Surgery 2016;160:1582-90.)*

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THYROID CANCER is the most common of the endocrine malignancies and accounts for 1% of all cancers.[1] In contrast to their undifferentiated counterparts, well-differentiated thyroid carcinomas (WDTCs) carry an overall good prognosis and can be divided into 2 different subgroups: a large majority that are treated effectively by operation followed by ^{131}I radioactive iodine (RAI) ablation and do not cause patients' death, and a minority that follow a more aggressive clinical course. Indeed, 5–10% of WDTC develop regional recurrences or distant metastases,[2-4] and 26–60% of those recurrences or metastases become refractory to RAI therapy,[5] which may very well lead to a fatal outcome.[2]

The success of RAI therapy is due to the almost unique ability of thyroid follicular cells to take up iodine, a process that is mediated by the sodium iodide symporter (NIS), codified by the Sl5a5c gene (solute carrier family 5).[6] Mammalian target of rapamycin (mTOR) is a downstream effector of the PI3K/Akt pathway that can be activated as part of the PI3K cascade and by other stimuli, such as growth factors, nutrients, energetic balance, stress signals, and signaling pathways such as MAPK.[7] The mTOR pathway is upregulated in a variety of neoplasias and in some of these neoplasias, pmTOR pathway is associated with a more aggressive behavior, including an increased tendency to metastasize and poor prognosis.[8,9]

Our group and others have observed an upregulation of the mTOR pathway in thyroid cancers, in comparison to the normal, adjacent, nonneoplastic tissue, through the overexpression of pmTOR and its downstream effectors, particularly in papillary thyroid cancer (PTC).[10,11] In the aforementioned studies, the contribution of the mTOR pathway activation for tumor behavior and patient prognosis was not addressed.

In models of thyroid cancer (cell lines and animal models), inhibition of the mTOR pathway caused a decrease in cell proliferation and tumor progression (decreased cell proliferation, motility, and invasion), decreased formation of distant metastases,[11,12] and was also able to upregulate the expression of NIS and to increase RAI uptake.[13,14] Moreover, in thyroid cell lines, the mTOR pathway seems to play an important role in iodine metabolism, both in normal as well as in malignant thyroid tissues. Inhibition of the mTOR pathway by rapamycin caused an increase of NIS expression and therefore a greater uptake of iodine.[13,14]

In light of all this information, we hypothesized that upregulation of the mTOR pathway may be associated, in malignant thyroid neoplasms to clinical aggressiveness, as well as with NIS downregulation, and to resistance to therapy. Because mTOR inhibitors have already been approved by the US Food and Drug Administration for the treatment of other malignancies,[7] it seems logical to try to understand the role of the mTOR pathway in thyroid cancers.

Following the aforementioned assumptions, we studied the status of the mTOR pathway (using 2 readouts: pmTOR and pS6) in a series of 191 cases of PTC and looked for associations with relevant clinicopathologic features, prognosis, and NIS expression.

MATERIALS AND METHODS

Patient tissue samples. A total of 191, formalin-fixed, paraffin-embedded, representative tissue samples from PTCs were collected from the files of the Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP, Porto, Portugal) corresponding to 191 patients followed in 2 university hospitals in Portugal; in 118 cases, we had follow-up data. Frozen material was available from 46 cancers that were divided into 2 equal parts: 1 part was conserved at −80°C, while the other part was formalin fixed and paraffin embedded for routine histology.

The histology of all tumors samples was reviewed (CE, ER, MSS) according to the criteria of the World Health Organization.[15] Epidemiologic, clinical, and molecular data of the 191 cases are summarized in Table I. The number of ^{131}I treatments varied from 1 to 5 treatments (mean 1.9), and the cumulative total dose of RAI was between 30 and 1,146 mCi (mean 251 mCi). All the procedures described in this study were approved by the respective ethical boards and are in accordance with national and institutional standards.

DNA extraction, PCR, and Sanger sequencing. DNA extraction from formalin-fixed, paraffin-embedded tissues was performed from 10 μm sections after careful microdissection. DNA extraction was performed using a tissue DNA kit (ULTRAprep; AHN Biotecnologie, Nordhausen, Germany) following the manufacturer's instructions. The genetic characterization (gene amplification and sequencing) of part of the tumors regarding BRAF,^{V600E} NRAS, RET/PTC, and TERT promoter mutations had been reported previously; mutations were screened as described formerly.[16-20]

Immunohistochemistry. Immunohistochemistry was performed as described previously.[11] Briefly,

Table I. Epidemiologic, histologic, and clinical data of patients included in the study

	Total (%)	*cPTC*	*fvPTC*	*Other variants*
Age, *n*	186	119	47	20
<45 y	99 (53)	66 (55)	22 (47)	11 (55)
≥45 y	87 (47)	53 (45)	25 (53)	9 (45)
Sex, *n*	190	121	48	21
Female	155 (82)	98 (81)	42 (88)	15 (71)
Male	35 (18)	23 (19)	6 (12)	6 (29)
Tumor size, *n*	181	114	47	20
<2 cm	69 (38)	43 (38)	18 (38)	8 (40)
≥2 cm	112 (62)	71 (62)	29 (62)	12 (60)
Tumor capsule, *n*	181	114	46	21
Present	88 (49)	46 (40)	33 (72)	9 (43)
Tumor capsule invasion, *n*	82	42	33	7
Yes	67 (82)	37 (88)	23 (70)	7 (100)
Extrathyroid extension, *n*	177	112	45	20
Present	76 (43)	53 (47)	12 (27)	11 (55)
Multifocality, *n*	182	114	48	20
Multifocal	75 (41)	53 (47)	16 (33)	6 (30)
Lymphocytic infiltrate, *n*	183	116	47	20
Present	113 (62)	81 (70)	20 (43)	12 (60)
Lymph node metastases, *n*	153	100	36	17
Present	62 (41)	44 (44)	13 (36)	5 (29)
Vascular invasion, *n*	178	112	46	20
Present	63 (35)	46 (41)	10 (22)	7 (35)
Tumor margins, *n*	117	77	29	11
Infiltrative	82 (70)	60 (78)	14 (48)	8 (73)
Distant metastases, *n*	120	81	29	10
Present	18 (15)	10 (12)	5 (17)	3 (30)
Staging (AJCC), *n*	107	73	26	8
I	66 (62)	47 (65)	15 (57)	4 (50)
II	6 (6)	3 (4)	3 (12)	0 (0)
III	25 (23)	20 (27)	3 (12)	2 (25)
IV	10 (9)	3 (4)	5 (19)	2 (25)
One y disease free, *n*	117	78	29	10
No	50 (43)	34 (4)	10 (35)	6 (60)
Disease free (end of follow-up), *n*	118	79	29	10
No	45 (38)	33 (4)	9 (31)	3 (30)
Deaths, *n*	121	81	29	11
Yes	5 (4)	2 (2.5)	2 (6.9)	1 (9.1)
BRAF, *n*	189	122	46	21
WT	112 (59)	62 (51)	37 (80)	13 (62)
V600E	77 (41)	60 (49)	9 (20)	8 (38)
NRAS, *n*	180	117	43	20
WT	171 (95)	116 (99)	39 (91)	16 (80)
Mutation	9 (5)	1 (1)	4 (9)	4 (20)
TERT promoter, *n*	166	106	43	17
WT	158 (95)	100 (94)	41 (95)	17 (100)
Mutation	8 (5)	6 (6)	2 (5)	0 (0)
RET/PTC rearrangement, *n*	69	40	19	10
Absent	59 (86)	32 (80)	18 (95)	9 (90)
Present	10 (14)	8 (20)	1 (5)	1 (10)

y, Years; *AJCC*, American Joint Committee on Cancer; *WT*, wild type.

sections were subjected to heat-induced antigen retrieval in 1 mM EDTA (pH 9.0) for the anti-phospho-S6 Ser235/236 antibody (Cell Signaling Technology, Danvers, MA, 1:400) and in 10 mM sodium citrate buffer (pH 6.0) for pmTOR Ser2448 (Cell Signaling Technology, 1:100). Endogenous

Table II. Distribution of pmTOR and pS6 scores in the series

	pmTOR		*pS6*	
Score	*Frequency*	*%*	*Frequency*	*%*
0	36	20	52	35
1	10	6	20	14
2	19	11	17	12
3	11	6	13	9
4	18	10	12	8
6	22	12	13	9
8	3	2	4	3
9	21	12	7	5
12	37	21	8	5
Total	177	100	146	100

peroxidase activity was blocked with 3% hydrogen peroxide and nonspecific binding with Large Volume Ultra V Block reagent (Thermo Scientific/Lab Vision, Waltham, MA). Sections were then incubated overnight at 4°C with the primary antibodies. Detection was performed with a labeled, streptavidin-biotin immunoperoxidase detection system (Thermo Scientific/Lab Vision) followed by 3,3′-diaminobenzidine (Dako, Glostrup, Denmark) reaction and counterstained with hematoxylin.

Evaluation of the immunostaining was done according to our previous work.[11] Slides were evaluated by 2 distinct observers and scored semiquantitatively in terms of percentage of stained tumor cells (0, <5%; 1, 5–25%; 2, 25–50%; 3, 50–75%; 4, >75%) and staining intensity (0, negative; 1, weak; 2, intermediate; 3, strong). An immunohistochemical score was calculated by multiplying the proportion of positive cells by the intensity of the staining, with 12 as a maximum score. The distribution of cases within the score is summarized in Table II.

The cellular localization was also evaluated as membrane and/or cytoplasmic and/or nuclear. To determine the predictive value of pmTOR for distant metastases, the following cut-off was based on the score: negative and very low expression scores (0, 1, and 2) were considered negative, while values of score ≥3 were considered positive. Slides were observed in an Axioskop 2 Zeiss microscope (Carl Zeiss, Jena, Germany) with photographs acquired using Nikon DS-L1 camera (Nikon, Tokio, Japan) in 100X and 400X magnifications.

RNA extraction and reverse transcription. Total RNA was extracted from PTCs in which frozen samples were available ($n = 46$) using a commercial kit (Trizol; Thermo Scientific/GIBCO) according to the manufacturer's protocol. RNA was quantified by spectrophotometry, and its quality was checked by analysis of 260/280 nm and 260/230 nm ratios. For cDNA preparation, 1 μg of total RNA was reverse transcribed using a first strand cDNA synthesis kit (RevertAid; Thermo Scientific/Fermentas).

Real-time PCR. Reverse transcription products were amplified for the Sl5a5c by qPCR (Integrated DNA Technologies [IDT], Leuven, Belgium, no. HS.PT.56a.40789288) using a PCR Master Mix (TaqMan; Applied Biosystems, Foster City, CA) with the TBP gene (TATA-binding protein) as endogenous control (Applied Biosystems, no. 4326322E-0705006). The ABI PRISM 7500 Fast Sequence Detection System (Applied Biosystems) was used to detect the amplification level and was programmed to an initial step of 20 seconds at 50°C, 10 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. For each sample, TBP and NIS amplifications were done in triplicate using 1 μl of cDNA (~25 ng). The relative quantification of target genes was determined using the $2^{-\Delta}$CT method. Similar efficiencies of both assays were confirmed using Livak's Linear Regression Method (slope −0.4).[21]

Patient follow-up. Patients were treated and followed in accordance with the international protocols available at the time. Data regarding the number of radioiodine treatments and cumulative activity were retrieved from hospital records. Patients were considered as being disease free at the end of follow-up if they had undetectable stimulated thyroglobulin (in the absence of thyroglobulin antibodies) and no evidence of the disease on radiographic or radionuclide imaging. The mean time of follow-up was 8 ± 6.7 years.

Statistical analysis. Statistical analysis was conducted with SPSS software (Version 21.00; SPSS, Inc, Chicago, IL). The results are expressed as mean ± standard deviation. Independent sample Student *t* test and Pearson correlation were used to evaluate association and correlation of pmTOR and pS6 expression with other variables. The predictive value of pmTOR expression and other factors (age, sex, tumor size, extrathyroid extension, vascular invasion, lymph node metastases, BRAF, and TERT promoter mutations) for distant metastases were assessed using univariate and multivariate logistic regression models.

RESULTS

The expression of pmTOR and pS6 were negative in 20.3% and 35.6% of the cases,

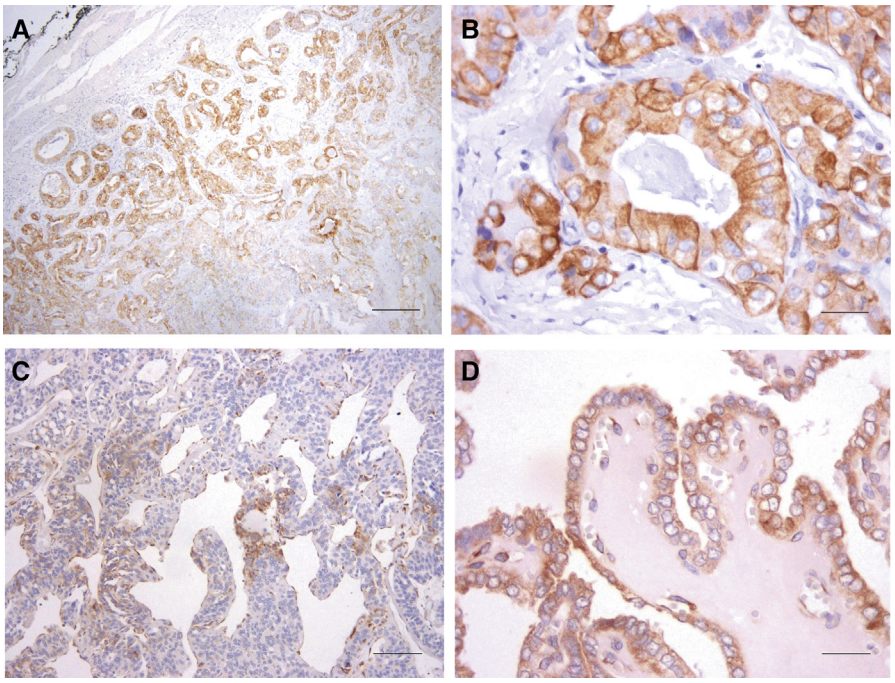

Fig. Expression of pmTOR (*A*, *B*) and pS6 (*C*, *D*) in PTC. pmTOR expression is more concentrated and intense in the periphery and the invasive front of the tumor (*A*). In a higher magnification, pmTOR expression is located in the cytoplasm and cytoplasmic membrane (*B*). pS6 expression is heterogeneous within the tumor (*C*) without a specific pattern; its location in the cell is only cytoplasmic and diffuse (*D*). *Bar* 100 μm. (Color version of this figure is available online.)

respectively (Table II). Among the group of cases classified as positive for pmTOR expression, most had high (9–12; 33%) or intermediate (3–8; 30%) scores. In the group of cases classified as positive for pS6 expression, most presented with intermediate (3–5; 29%) or low (1–2; 25%) score values (Table II). The distribution and intensity of pmTOR staining within the tumor was heterogeneous in the majority of cases; pmTOR staining was more concentrated and/or stronger in the invasive front of the tumors and located mainly in the cytoplasm and cellular membrane of the neoplastic cells (Fig, *A* and *B*). pS6 expression was exclusively cytoplasmic, displaying a topographic heterogeneous distribution without any specific pattern (Fig, *C* and *D*).

Relationship between the expression of pmTOR and pS6 and clinicopathologic features. Greater pmTOR expression was associated with absence of a tumor capsule ($P = .01$), presence of distant metastases ($P = .05$), persistence of disease (one year disease-free status and disease-free status at the end of follow-up) ($P = .05$), and NRAS mutation ($P = .04$) (Table III). Furthermore, greater pmTOR expression was also correlated with a greater number of ^{131}I therapies (r[102] 0.2, $P = .02$), greater cumulative dose of RAI (r[100] 0.3, $P = .01$), and a lesser expression of NIS (r[44] −0.3, $P = .03$).

Analyzing the 2 main variants of PTC, cPTC, and the follicular variant of PTC (fvPTC) independently, greater pmTOR expression was associated with absence of a tumor capsule ($P = .02$), a TERT promoter wild type (WT) ($P = .01$), and persistence of disease at the end of follow-up ($P = .05$) in the cPTC group; no statistically significant differences were found in the group of cases of fvPTC concerning pmTOR expression.

A logistic regression model was performed for factors associated with distant, blood-borne metastases (Table IV). A total of 18 patients (15%) developed distant metastases detected during follow-up; the metastases were located in lung ($n = 11$), bone ($n = 5$), lung and bone ($n = 1$), and brain ($n = 1$). Cases from male patients (odds ratio [OR] 3.7; $P = .02$) with vascular invasion (OR 5.2; $P = .01$) and positive pmTOR expression (OR 8.2; $P = .01$) had a greater risk of developing distant metastases. When all the features associated with distant metastases in the univariate model were introduced in a multivariate regression model, positive pmTOR expression became the only independent predictive factor of distant metastases (Table IV). Using the same statistic model, we

Table III. Summary of clinicopathologic and molecular associations with pmTOR and pS6 expression

	Frequencies	*pmTOR mean expression*	P *value*	*Frequencies*	*pS6 mean expression*	P *value*
Tumor capsule	Present (n = 83)	*4.4 ± 4.4*	*.01*	*(n = 60)*	*3.7 ± 3.6*	*.01*
	Absent (n = 85)	*6.2 ± 4.4*		*(n = 77)*	*2.2 ± 3.1*	
Extrathyroid invasion	Yes (n = 70)	5.1 ± 4.4	.8	*(n = 55)*	*1.6 ± 2.6*	*.001*
	No (n = 94)	5.3 ± 4.6		*(n = 78)*	*3.5 ± 3.6*	
Lymphocytic infiltrate	Present (n = 102)	5.5 ± 4.5	.8	*(n = 82)*	*2.0 ± 2.8*	*<.001*
	Absent (n = 69)	5.3 ± 4.4		*(n = 58)*	*4.2 ± 3.9*	
Tumor margins	Infiltrative (n = 72)	4.2 ± 4.2	.4	*(n = 53)*	*1.5 ± 2.6*	*.05*
	Well defined (n = 32)	3.4 ± 3.9		*(n = 20)*	*2.9 ± 2.7*	
Distant metastases	Yes (n = 14)	*5.93 ± 3.91*	*.05*	(n = 11)	2.8 ± 2.9	.2
	No (n = 92)	*3.61 ± 4.1*		(n = 64)	1.6 ± 2.6	
One y disease free	Yes (n = 61)	*3.3 ± 3.5*	*.05*	(n = 44)	1.7 ± 2.7	.6
	No (n = 42)	*4.9 ± 4.7*		(n = 29)	2.0 ± 2.8	
Disease free (end of follow-up)	Yes (n = 67)	*3.4 ± 3.6*	*.05*	(n = 48)	1.8 ± 2.7	1.0
	No (n = 37)	*5.1 ± 4.8*		(n = 26)	1.8 ± 2.6	
BRAF	WT (n = 101)	5.4 ± 4.5	.9	*(n = 81)*	*3.5 ± 3.7*	*.01*
	V600E (n = 75)	5.3 ± 4.5		*(n = 64)*	*1.9 ± 2.9*	
NRAS	WT (n = 158)	*5.2 ± 4.4*	*.04*	*(n = 132)*	*2.6 ± 3.3*	*.001*
	Mut (n = 9)	*8.3 ± 5.0*		*(n = 9)*	*6.7 ± 3.7*	

We did not find any significant associations between pmTOR and pS6 expression and: sex, tumor size, tumor capsule invasion, tumor multifocality, tumor size, vascular invasion, lymph node metastases, TERT promoter mutation and RET/PTC rearrangements. Values in italics are statistically significant results.
y, Year; *WT*, wild type; *Mut*, mutation.

were also able to observe that positive pmTOR expression is not a risk factor for the development of lymph node metastases.

Greater pS6 expression was associated with the presence of a tumor capsule (P = .01), absence of extrathyroid invasion (P = .001), well-defined tumor margins (P = .05), absence of lymphocytic infiltrate (P < .001), WT BRAF status (P = .01), and NRAS mutation (P = .001) (Table III). When the subgroup of cPTC was analyzed independently, only the association of greater pS6 expression and absence of extrathyroid invasion remained statistically significant (P = .004). In the fvPTC group, the associations between greater pS6 expression and absence of lymphocytic infiltrate (P = .003), BRAF WT (P < .001), and NRAS mutation (P = .02) remained significant. There was no significant correlation between pmTOR and pS6 expression.

DISCUSSION

In the present study, we have found interesting results regarding the role played by the mTOR pathway as well as conflicting data regarding the difference of the 2 readouts we used, pmTOR and pS6. We observed that pmTOR expression appears to be an indicator of tumor aggressiveness in PTC. Its expression was associated with absence of a tumor capsule, presence of distant metastases, persistence of disease, RAS mutation (Table III), and it correlated with a greater number of RAI therapies, greater cumulative dose of RAI, and with a lesser NIS expression.

In contrast, pS6 expression was associated with less aggressive pathologic features, such as presence of a tumor capsule, absence of extrathyroid extension, well-defined tumor margins, and BRAF WT status (Table III). Despite being members of the same pathway, we did not find a correlation between the expression of those 2 markers, indicating that, in our series, the expression of pmTOR and pS6 are not linked to each other.

Activation of mTOR was associated with distant metastases and persistence of disease in PTC. The only available evidence that mTOR may be implicated in the ability of thyroid cancer cells to metastasize is a mouse model that develops thyroid cancer and distant metastases spontaneously. In this specific model, blockade of the mTOR pathway totally prevented the formation of distant metastases, but not tumor formation,[12] indicating that mTOR activation may be more important for tumor progression than for tumor initiation.

Due to the clinical relevance of distant metastases, we performed a multivariate logistic regression evaluating the clinicopathologic and molecular features associated with distant metastases. Male sex, presence of vascular invasion, and a positive pmTOR expression were significant predictors of

Table IV. Predictive factors for distant metastases

	Distant metastases n = *120*				
		Univariate analysis		*Multivariate analysis*	
	Presence %	*OR (95% CI)*	P *value*	*OR (95% CI)*	P *value*
Total	18 (15.0)				
Sex					
Female	10 (10.6)	1		1	
Male	8 (30.8)	3.7 (1.3–10.8)	*.02*	3.3 (0.7–15.4)	.1
Vascular invasion					
No	6 (7.3)	1		1	
Yes	9 (29.0)	5.2 (1.7–16.2)	*.005*	3.7 (0.9–14.7)	.06
pmTOR					
Negative	2 (3.6)	1		1	
Positive	12 (25.5)	8.2 (1.7–38.5)	*.01*	18.2 (2.1–157.9)*	*.01*

*All the variables considered significant for univariate analysis were included in the multivariate model.
We found no significant predictive ability of age, tumor size, extrathyroid extention, lymph node metastases, or BRAF or TERT promoter mutations for distant metastases on univariate and multivariate analysis. Values in italics are statistically significant results.
OR, Odds ratio.

distant metastases in a univariated model. When all the features associated with distant metastases in the univariated model were included in the regression, positive pmTOR expression was the only significant predictor (Table IV). Interestingly, positive pmTOR expression was not a predictor for lymph node metastases, which are known to have much less prognostic impact than distant metastases in PTC,[3] thus revealing some specificity for the type of metastases.

The association of the mTOR pathway with essential steps in the metastatic cascade has already been observed in other tumor models in vitro[22-24] and in vivo.[25] In human tumor specimens, the mTOR pathway was associated with lymph node metastases in invasive ductal breast carcinoma,[25] with persistence of disease and poor prognosis in gastric cancer,[26] and with poorer prognostic characteristics in cutaneous melanoma.[9] Once activated, pmTOR can associate with a subset of different proteins, forming 2 distinct complexes with distinct biologic roles: mTORC1 and mTORC2.[7,27] Although both mTOR complexes are implicated with cell motility, invasion, and metastatic ability, mTORC2 is more often correlated with these tumor features and worse prognosis.[23,24,26] In models of breast and renal cell carcinoma, only inhibition of mTORC2 and not mTORC1 was able to inhibit cell motility, invasion, and metastases.[23,24]

In our study, we did not obtain enough information to discriminate which mTOR complex(es) is(are) contributing to tumor aggressiveness, because the antibody we used is directed to pmTOR at Ser2448, and phosphorylation at this site is not exclusive of a specific mTOR complex.[27]

The distinct associations of the expression of pmTOR and pS6 with clinicopathologic data, molecular features, and prognosis, as well as the lack of correlation between their expression (as observed in invasive breast cancer[28]) led us to hypothesize that activation of pmTOR preferentially leads to the assembly of mTORC2 instead of mTORC1.

Further studies are needed to prove our hypothesis, but the findings of our study support this hypothesis. We know that mTORC2 phosphorylates Akt at Ser473 at the cellular membrane,[29] and in the majority of our cases, we showed that pmTOR displays a membrane staining and a preferential location in the invasive front of the tumor. Furthermore, another study demonstrated that SIN1 (another critical factor in the mTORC2 complex) was overexpressed in PTCs displaying more aggressive histologic features,[30] suggesting a preponderant role of mTORC2 toward aggressiveness in thyroid cancer.

Besides being implicated in distant blood-borne metastases, we were also able to show an inverse correlation between pmTOR and NIS expression in PTC. This correlation fits with previous in vitro results in which blockade of the mTOR pathway in cell lines derived from thyroid cancers caused an increase in NIS expression and also in RAI uptake.[13,14] Although statistically significant, this correlation was weak and was based on a relatively small number of cases (n = 46); further studies involving larger series are needed to validate these results.

Expression of pmTOR was also correlated directly with a greater number of ^{131}I therapies and thus with greater cumulative dose of RAI.

Because of these results, it may be important to explore the role of the mTOR pathway in the resistance to ^{131}I therapy in order to evaluate possible advantages of pharmacologic blockers of mTOR in PTC resistance to RAI therapy.

Regarding NRAS mutations, despite the small number of mutated cases, we observed a significant overexpression of pmTOR and pS6 in RAS-mutated cases compared to WT cases. RAS protein is able to activate PI3K, which then activates the mTOR pathway.[7,31] The results of the present study, as well as those reported previously by our group showing that medullary thyroid carcinomas with RAS mutation had greater activation of the mTOR pathway,[32] suggest that RAS mutations may be a trigger for the activation of the mTOR pathway in thyroid cancers derived from both follicular and parafollicular cells.

Limitations of the present study may explain the divergent results in comparison with previous reports[11] and those of others,[33] regarding associations between pmTOR and pS6 expression with clinicopathologic and molecular features. Ahmed et al[33] reported an association with older age (≥45 years) and higher tumor staging[33] that we did not find in our current series. Moreover, we also did not confirm our previous results[11] regarding pmTOR and pS6 overexpression in $BRAF^{V600E}$ cPTCs compared to $BRAF^{wt}$[11].

In the present study, we did not observe a difference between the expression of pmTOR and pS6 in cPTCs with distinct BRAF contexts ($BRAF^{wt}$ or $BRAF^{V600E}$). These discordant results may be due to methodologic differences of the 3 studies. Faustino et al[11] and Ahmed et al[33] evaluated pmTOR and pS6 expression in tissue microarrays (TMA), while in the present study, we used histologic sections. We observed that both pmTOR and pS6 have a very heterogeneous distribution within the tumor (especially pmTOR, being more concentrated in the tumor periphery and invasive front); the TMA evaluation may thus be inadequate, because the limited samples may be not representative of the overall tumor expression.

In conclusion, pmTOR seems to be a promising marker of the aggressiveness (distant metastases, persistence of disease, and refractory disease) in PTC. In order to develop a more effective therapeutic strategy, further studies are needed to understand exactly the biologic consequences of each of the 2 mTOR complexes in thyroid cancers, because they seem to play different roles in tumor progression and metastases.

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Chapter 4. Paper 2. mTOR pathway in papillary thyroid carcinoma: different contributions of mTORC1 and mTORC2 complexes to tumor behavior and *SLC5A5* mRNA expression

This chapter is presently a manuscript in preparation with the same title.

mTOR pathway in papillary thyroid carcinoma: different contributions of mTORC1 and mTORC2 complexes to tumor behavior and *SLC5A5*mRNA expression

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Abstract

mTOR pathway is overactivated in thyroid cancer (TC). Once activated, mTOR can lead to the assembly of two different complexes mTORC1 and mTORC2, with distinct downstream effectors: pS6 Ser235/236 and pAKT Ser473, respectively. TC treatment is based on surgery followed by therapy with radioactive iodine (RAI) which is uptaken by TC cells through the sodium iodide symporter (NIS) codified by the *SLC5A5* gene. In our previous study we observed that pmTOR expression was associated with tumor aggressiveness and therapy resistance in papillary thyroid carcinomas (PTCs). On the contrary, pS6 expression was associated with less aggressive clinicopathological and molecular features. The distinct behavior of the two markers led us to hypothesized, that mTOR activation could be contributing, in PTC, to a preferential activation of mTORC2 complex in detriment of mTORC1 complex.

We performed immunohistochemistry for pAKT Ser473 in a series of 182 PTCs previously characterized for pmTOR and pS6 expression. Furthermore, we analyzed the impact of each mTOR complex on *SLC5A5* mRNA expression, by treating a cell line derived from PTC with RAD001 (mTORC1 blocker) and Torin 2 (mTORC1 and mTORC2 blocker).

pAKT Ser473 expression was positively correlated with pmTOR expression and significantly higher in PTCs harboring *BRAF*V600E mutation than in *BRAF*WT PTCs. Moreover, pAKT Ser473 nuclear expression was significantly associated with the presence of distant metastases. Treatment of TPC1 cell line with RAD001 had no consequences on *SLC5A5* mRNA levels, whereas Torin2 caused a ~6fold increase of *SLC5A5* mRNA expression.

mTORC2 pathway is activated in PTCs and the nuclear translocation of its downstream effector pAKT Ser473 may play a major role in distant metastization. mTORC2 inhibition upregulates the expression of *SLC5A5* mRNA. Pharmacological inhibition of mTORC2 complex should be further addressed in the management of specific RAI resistant TC.

4.1 Introduction

Thyroid cancer (TC) is the most common endocrine neoplasia. Differentiated thyroid carcinoma (DTC) arises from thyroid follicular cells and represents more than 90% of all cases of TC. DTC comprises papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC) being the PTC the most prevalent type[1, 2]. PTC can be further subdivided in variants, the more prevalent being the so called classic PTC (cPTC) and the follicular variant of PTC (FVPTC)[1].

PTC carries a very good prognosis with a 10 years 93-95% survival, being treated with surgery followed by radioactive iodine (RAI). By poorly understood reasons, a subgroup of TC patients (10-15%) becomes resistant to RAI treatment[3] what could lead to a significant reduction of their 10-years survival [4]. The molecular mechanism behind this resistance relies, at least in part, in the loss NIS expression and/or function. NIS is codified by the *SLC5A5* gene, being normally expressed at the basolateral membrane of thyroid follicular cells. Usually, PTCs maintain NIS expression and function allowing the incorporation of ^{131}I that cause tumor cell death, a very efficient targeted radiotherapy[5].

mTOR pathway is overactivated in a variety of human neoplasias[6], including in TC[7-9]. It can be activated by diverse stimuli, such as growth factors, nutrients, energy, stress signals and other essential signaling pathways, such as PI3K and MAPK[6, 9, 10]. Once activated, mTOR can associate with different proteins forming two distinct complexes, mTORC1 and mTORC2. The complexes have different downstream effectors and physiological functions: mTORC1 effectors are S6K1 and 4EBP1 that participate in cellular growth, proliferation and survival, whereas mTORC2 phosphorylates PKC-α and AKT (Ser 473) and regulates the actin cytoskeleton of the cell and cell migration[6, 10].

A recent study of our group demonstrated that pmTOR is a marker of aggressiveness in PTC: its expression is associated with aggressive clinicopathological features, including distant metastases, resistance to ^{131}I therapy and, consequently, worse prognosis[11]. In the same study, we observed that pS6 expression was associated with clinicopathological features of low aggressiveness and we did not find a significant correlation between pmTOR and pS6 expression

in each tumor[11]. The absence of correlation between the two proteins and the divergent behavior presented by them led us to hypothesized that, in PTC, the activation of pmTOR might be contributing preferentially to the formation of mTORC2 complex, and consequently to AKT activation[11] (pAKT Ser473) as it has been observed in other tumor models[12,13,14,15]. pAKT is upregulated in PTCs[7-9], but its role in PTCs' clinical behavior and resistance to therapy needs to be further explored.

Previous studies showed that when mTOR pathway is inhibited, NIS expression increases; however, such studies only explored the role of mTORC1 complex[16, 17]. As far as we are aware, mTORC2 role on *SLC5A5* expression was not previously studied. So far, it is known that dual inhibition of mTORC1 and mTORC2 complexes by Torin2 in TC models causes a decrease in cell growth [18, 19] and inhibits metastization [19]. Still, the impact of Torin2 on *SLC5A5* mRNA expression or NIS protein function was not addressed.

It is also well established that *BRAF*V600E mutation, the most prevalent mutation in PTCs[20], has a negative impact on NIS expression and targeting to the membrane[21, 22], but this effect does not seem to be mediated by MAPK[22]. Alternative molecular links between *BRAF*V600E mutation and NIS expression have been proposed[23-26]. An alternative mechanism could be mediated by mTOR, since *BRAF*V600E seems to contribute to an over-activation of mTOR pathway in PTCs[9] and mTOR pathway over-activation has a negative impact on NIS expression and function[16, 17].

In this study, we intended to understand the relevance of mTORC2 complex activation in PTC, by exploring the role of pAKT Ser473 in PTC clinical behavior and the response of a TC cell line to Torin2 dual inhibition of mTORC1 and mTORC2 complexes.

4.2 Materials and Methods

Patient tissue samples

One hundred and eighty-two formalin-fixed, paraffin embedded representative tissue samples from PTCs were collected from the files of the Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP, Porto, Portugal), corresponding to 182 patients followed in two university hospitals in Portugal. In 115 cases, we had access to follow-up data. The histology of all tumors samples was revised (CE, ER, MSS) according to the World Health Organization criteria [27]. Epidemiological, clinical, and molecular data of the 182 cases are summarized in Table 1. The number of ^{131}I treatments varied between 1 to 5 (mean 1.8) and cumulative dose of RAI totalized values between 30 and 1146 mCi (mean 245.2 mCi). All the procedures described in this study were approved by the respective ethical boards and are in accordance with national and institutional standards.

Patient's follow up

Patients were treated and followed in accordance with the international protocols available at the time. Data regarding the number of radioiodine treatments and cumulative activity were retrieved from hospital records. Patients were considered as being disease free at the end of follow-up if they had undetectable stimulated thyroglobulin (in the absence of thyroglobulin antibodies) and no imagiological evidence of the disease. The mean time of follow up was 8±6.8 years. For statistical analysis, we defined the category "additional treatments", in which we included other treatment modalities in addition to radioiodine, including extra surgery, external beam irradiation, and treatment with tyrosine kinase inhibitors.

Immunohistochemistry

Immunohistochemistry was performed as previously described[9]. Briefly, sections were subjected to heat-induced antigen retrieval in 10 mM sodium citrate buffer (pH6.0). Endogenous peroxidase activity was blocked with 3% of H2O2 and nonspecific binding with Large Volume Ultra V Block reagent (Thermo Scientific/Lab Vision, Waltham, MA, USA). Sections were then

incubated overnight at 4ºCwith anti pAKT Ser473 antibody (clone 736E11) (Cell Signaling Technology, Danvers, USA) (1:50).

The detection was performed with a labeled streptavidin-biotin immunoperoxidase detection system (Thermo Scientific/Lab Vision, Waltham, MA, USA) followed by 3,3'-diaminobenzidine (Dako, Glostrup, Denmark) reaction and counterstained with hematoxylin.

The immunostaining evaluation was done according to our previous work [9]. Slides were evaluated by two observers and semiquantitatively scored in terms of percentage of tumoral stained cells (0 - <5%; 1 - 5 to 25%; 2 - 25-50%; 3 - 50-75%; 4 - >75%) and staining intensity (0 - negative; 1 - weak; 2 - intermediate; 3 - strong). An immunohistochemical score was calculated by multiplying the proportion of positive cells by the intensity of the staining, with 12 as maximum score. The distribution of cases within the scores is summarized in Table 2. The cellular localization was also evaluated as membrane and/or cytoplasmic and/or nuclear. To be considered positive for nuclear expression, tumors must display pAKT Ser473 immunostaining in at least 5% of tumor cells. Slides were observed in an Axioskop 2 Zeiss microscope. Representative slides were scanned using DSight Viewer (Menarini) and photographs were obtained through snapshots from the DSight Viewer Software (Menarini). From the 182 cases characterized for pAKT Ser473, 170 have been previously characterized for pmTOR Ser2448 and 141 for pS6 Ser235/236[11].

DNA extraction, PCR and Sanger sequencing

The genetic characterization (gene amplification and sequencing) of the tumors regarding *BRAF*, *NRAS*, *RET/PTC* and *TERT* promoter (*TERTp*) mutations were screened as previously described [28-32] and part had been previously reported[11].

Cell lines and treatments with RAD001 and Torin2

TPC1 1 cell line used in this study is from papillary thyroid carcinoma origin. It was already characterized at the molecular and genotypic level, and cell line harbors *RET/PTC*1 rearrangement and *TERT*p mutation (-124G>A)[9, 32]. Cell line was maintained in RPMI supplemented with antibiotics 1% (vol/vol) Pen Strep and 0.5% fungizone (vol/vol) (Biowest, Nuaillé, France) and

10% (vol/vol) of fetal bovine serum (FBS) (GIBCO, Thermo Fisher Scientific Waltham, MA USA). Cells were grown in a humidified incubator with 5% C02 at 37ºC.

For treatment purposes, cells were plated in six wells plates, ($1x10^5$ cells per well), 24 hours latter cells were treated with RAD001 (20nM) or Torin2 (450nM) (Selleckchem, Houston, TX, USA). Treatments lasted for 60 hours and 72 hours. After that cells were lysed in RIPA buffer (supplemented with protease and phosphatase inhibitors) for western blot analysis or in Trizol for RNA extraction.

RNA extraction, reverse transcription and real time PCR

Total RNA was extracted from TPC 1 cells using a Trizol commercial kit (Thermo Scientific/GIBCO, Waltham, MA, USA) according to the manufacturer's protocol. RNA was quantified by spectrophotometry, and its quality was checked by analysis of 260/280 nm and 260/230 nm ratios. For cDNA preparation, 1µg of total RNA was reverse transcribed using the RevertAid first strand cDNA synthesis kit (Thermo Scientific/Fermentas, Waltham, MA, USA).

Reverse transcription products were amplified for *SLC5A5* by qPCR (IDT:Integrated DNA Technologies, Leuven, Belgium; no. HS.PT.56a.40789288) using TaqMan PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with TBP gene (TATA-binding protein) as endogenous control (Applied Biosystems; no. 4326322E-0705006). The ABI PRISM 7500 Fast Sequence Detection System (Applied Biosystems, Foster City, CA, USA) was used to detect the amplification level and was programmed to an initial step of 20 seconds at 50 °C, 10 min at 95° C, followed by 40 cycles of 95°C for 15 seconds and 60 ° C for 1 min. For each sample, TBP and *SLC5A5* amplifications were done in triplicate using 1µl of cDNA (~25ng).The RNA extraction and *SLC5A5* expression from the 31 PTCs in which frozen samples were available had been previously reported[11].

The relative quantification of target genes was determined using the $2^{-\Delta\Delta}$CT method. Similar efficiencies of both assays were confirmed using Livak's Linear Regression Method [33] (slope -0.4).

Western blot analysis

Cells were lysed in RIPA buffer supplemented with phosphatase and protease inhibitors. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). The primary antibodies were pS6 Ser235/236, S6, pAKT Ser473, AKT, (1:1000) all from Cell Signaling Technology (Danvers, MA). Protein was detected using a horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and a luminescence system (Perkin-Elmer). For protein loading control, membranes were incubated with an anti actin (Santa Cruz Biotecnology, Santa Cruz, CA) antibody. Protein expression was quantified using the Bio-Rad Quantitaty One 1-D Analysis software (Bio-Rad Laboratories, Inc., Hercules, CA). The levels of phosphorylated proteins: pS6 Ser235/236 and pAKT Ser473 were normalized by the levels of their corresponding total protein (total S6 and AKT). The levels of expression of phosphorylated proteins and their corresponding total protein were evaluated in the same gel, furthermore, the antibodies used for the total proteins recognize all forms of the phosphorylated proteins.

Statistical analysis

Statistical analysis was conducted with SPSS version 21.00 (SPSS Inc). The pAKT Ser473 expression results are expressed as mean ± standard deviation. Independent samples Student's *t* test was used to evaluate possible associations between pAKT Ser 473 expression and clinicopathological and molecular features. Pearson Correlation was used to evaluate the correlation between pAKT Ser473 and pmTOR Ser2448 and pS6 Ser235/236 expression. Chi-square test was used to evaluate possible associations between pAKT Ser473 nuclear expression and clinicopathological and molecular features. Independent samples Student's *t* test was also used to compare protein expression (analyzed by western blot) between groups. Results were considered statistically significant at $P \leq 0.05$.

4.3 Results

pAKT Ser473 immunoexpression

The expression of pAKT Ser473 was negative in 49.5% of the cases and the 50.5% of the positive cases were distributed throughout the score values (Table 2). In the group of positive cases, the immunostaining was found in the cytoplasm in 40/92 of the cases and simultaneously in the cytoplasm and nucleus in 52/92 of the cases.

Among the positive cases, pAKT Ser473 was more intense and preferentially located at the invasive front in 44% of the tumors. Once in the tumor's periphery, pAKT Ser473 was more frequently located in the nucleus (67.6% of the cases with pAKT Ser473 in the invasive front of the tumor, displayed nuclear staining) (Figure 1).

Relationship between the pAKT Ser473 expression and clinicopathological and molecular features.

pAKT Ser473 expression was positively correlated with pmTOR expression [r(168)=0.2, *P*=0.02) but not with pS6 expression[r(139)=0.02, *P*=0.8).

pAKT Ser473 was significantly more expressed in PTCs harboring *BRAF*V600E mutation compared to wild type (*P* = 0.04) (Table3), this significant association was maintained in the cPTC group but was not observed in the FVPTC group. pAKT Ser473 expression, in the overall PTC group or in cPTC or FVPTC group, was not associated with: age, tumor size, tumor capsule, multifocality, lymphocytic infiltrate, vascular invasion, lymph node metastases, tumor margins, distant metastases, staging, *NRAS* and *TERT*p status, number of ^{131}I therapies or cumulative dose of radioactive iodine, additional treatments, disease-free status at one year and disease-free status at the end of follow-up.

When cases were divided regarding pAKT Ser473 nuclear expression (presence or absence) we observed that cases presenting distant metastases displayed pAKT Ser473 in the nucleus more often compared to the cases without distant metastases (*P*=0.04) (Table4). We did not find any significant associations between pAKT Ser473 nuclear expression and other clinicopathological or molecular features (all PTCs, cPTC or FVPTC subgroups)

Regulation of *SLC5A5* expression by mTOR pathway: contribution of mTORC1 and mTORC2 complexes

To study the role of both mTORC1 and mTORC2 complexes on *SLC5A5* expression, we performed treatments of TPC1 cell line with RAD001 (mTORC1 inhibitor) and Torin 2 (mTORC1 and mTORC2 dual inhibitor) for 60 and 72 hours.

First, we confirmed the efficacy of the drugs by addressing pS6 expression as readout of mTORC1 activity and pAKT Ser473 as readout of mTORC2 activity. After 72hours of treatment, RAD001 caused an efficient down regulation of mTORC1 complex and did not affect the activity of the mTORC2 complex (significant decrease of pS6 expression and no differences in pAKT Ser473 expression) (Figure 2A and B). Additionally, Torin 2 treatment led to an efficient and simultaneous down regulation of mTORC1 and mTORC2 complexes (significant decrease of pS6 and pAKT Ser473 expression) (Figure 2A and B), these effects were also observed after 60 hours of treatment.

At 72h, RAD001 treatment did not affect *SLC5A5* expression, whereas Torin 2 caused a significant increase of *SLC5A5* mRNA expression (~6fold, *P*=0.02) (Table5 and Figure3). *SLC5A5* mRNA expression was not altered after 60h of treatment with both drugs (Table 5).

4.4 Discussion

The first aim of this work emerged from our previous study[11], and consisted in try to understand if pmTOR activation was conducting to a preferential formation of the mTORC2 complex in PTC. In the present study, we observed a positive and significant correlation between pmTOR and pAKT Ser473 expression (readout of mTORC2 activation), meaning that PTCs that expressed higher levels of pmTOR also expressed higher levels of pAKT Ser473. We also demonstrated that pAKT Ser473 nuclear expression is associated with the presence of distant metastases. The positive correlation between pmTOR and pAKT Ser473 and the significant association between pAKT Ser473 expression and distant metastization (that we also found in our previous work for pmTOR[11]) corroborates our hypothesis that, in PTC, mTOR activation is

leading to a preferential assembly of mTORC2 complex and its downstream effector pAKT Ser473, that seems to play a role in distant metastization.

Preferential formation of the mTORC2 complex was previously observed in other human malignancies, and is usually associated with increased cell motility[12-15]. In TC, both mTORC1 and mTORC2 complexes are overexpressed compared to normal tissues[9, 18], but the contribution of each complex to tumor behavior and prognosis is not fully understood. pAKT Ser473 is overexpressed in TC[7-9, 34], and its expression has been associated with metastization in other tumors[35-37], as well as, in animal models of TC[38, 39].

Our results, also point out the activation of pAKT Ser473 as an important step for TC distant metastization. We observed that pAKT Ser473 expression was associated with distant metastization only when we considered its nuclear expression. In fact, it seems that pAKT Ser473 nuclear translocation is of major importance to migration and distant metastization of TC. Vasko et al.,[8] demonstrated that pAKT Ser473 was expressed in the cytoplasm of PTC throughout the tumor, but the immunostaing was more intense and localized in the nucleus of cells located in the invasive regions. We also observed that when pAKT Ser473 staining was more concentrated in the invasive front of the tumor, it was preferentially located in the nucleus. Moreover, in an animal model of TC, pAKT Ser473 was localized primarily in the nucleus of cells from metastatic lesions, while in the primary tumors it was located in the cytoplasm and nucleus of cells, suggesting that pAKT nuclear distribution may be relevant to both initiation and sustaining metastasis[39].

In our series overall pAKT Ser473 expression was significantly higher in PTCs harboring *BRAF*V600E mutation compared to *BRAF*WT PTCs. In our previous study, we observed that *BRAF*V600E PTCs expressed similar levels of pmTOR but significantly lower levels of pS6 compared to *BRAF*WT PTCs[11], so it seems that PTCs harboring *BRAF*V600E mutation have a preferential activation of the mTORC2 complex in comparison to mTORC1.

It may seem controversial with the consistent observation that *BRAF*V600E mutation is not associated with distant metastization[40-42]. However, in our series, only the nuclear pAKT Ser473 expression is associated with distant metastization, suggesting that, nuclear translocation of

pAKT Ser473 is more important than its overall expression, regarding to distant metastization, which could explain these apparently discordant results.

Loss of NIS expression and function has been indicated as the molecular mechanism responsible for RAIR in TC[5]. Recent studies explored the role of mTOR pathway on NIS expression/function in rat thyroid cells[16] and in cell lines of TC (8505C, TPC1 and BCPAP)[17], both demonstrating that treatments with rapamycin, an mTORC1 inhibitor, was able to restore NIS expression and function in the majority of them [16, 17]. Since these works only evaluated the impact of mTORC1 on NIS expression and function, we were interested in exploring the role of mTORC2 in *SLC5A5 mRNA* expression. Albeit RAD001 caused a decrease on pS6 expression, it did not alter *SLC5A5* expression, as was previously observed in TPC1cell line[17]. Torin2 treatment caused a decrease of pS6 and pAKT Ser473 expression, and was also able to significantly increase *SLC5A5*mRNA expression, (Figure 2 and 3). The inhibition of mTORC2 complex revealed to be of major importance in the restoration of *SLC5A5*mRNA expression, high lightening its role as a potential therapeutic target.

This study demonstrated that mTORC2 pathway is activated in PTCs and that its downstream effector pAKT Ser473 nuclear translocation may play a major role in distant metastization. Thus, we considered that inhibition of mTORC2 complex should be further addressed in the management of specific RAIR TC.

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4.6 Figures/Figures legends

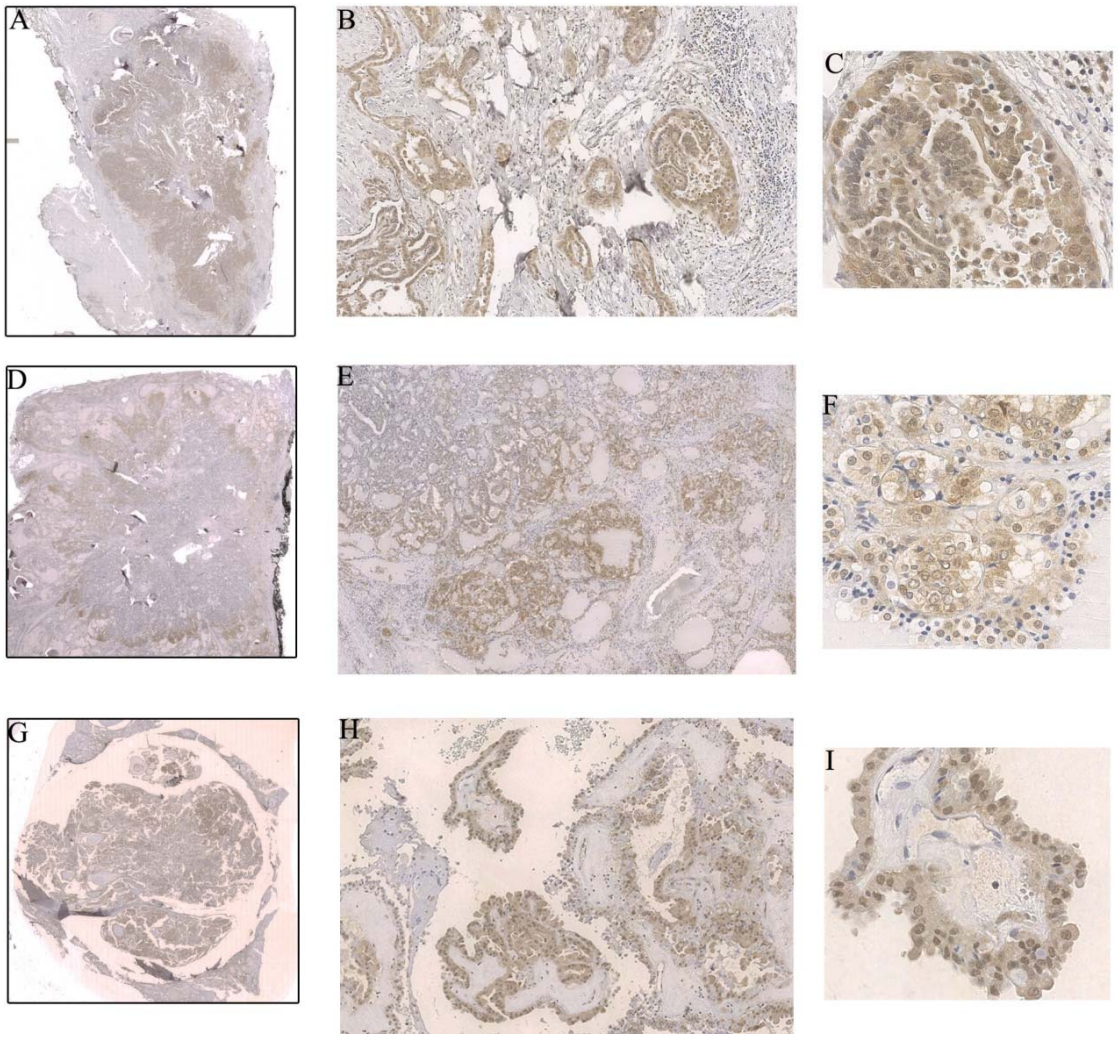


Figure 1. pAKT Ser473 immunoexpression in PTCs. **A, B, C)** Intensification of the immunostaining and pAKT Ser473 nuclear expression in the invasive front of a cPTC; A 0.44X, B 10X and C 40X magnification. **D, E, F)** Preferential pAKT Ser473 expression in the tumor periphery, another example on a cPTC. Note that, in this case, the nuclear translocation was not so intense compared to previous; D 0.44X, E 4X and F 40X magnification. **G, H, I)** strong and disseminated pAKT Ser473 nuclear expression in a hobnail variant of PTC; G 0.44X, H 10X and I 40X magnification.

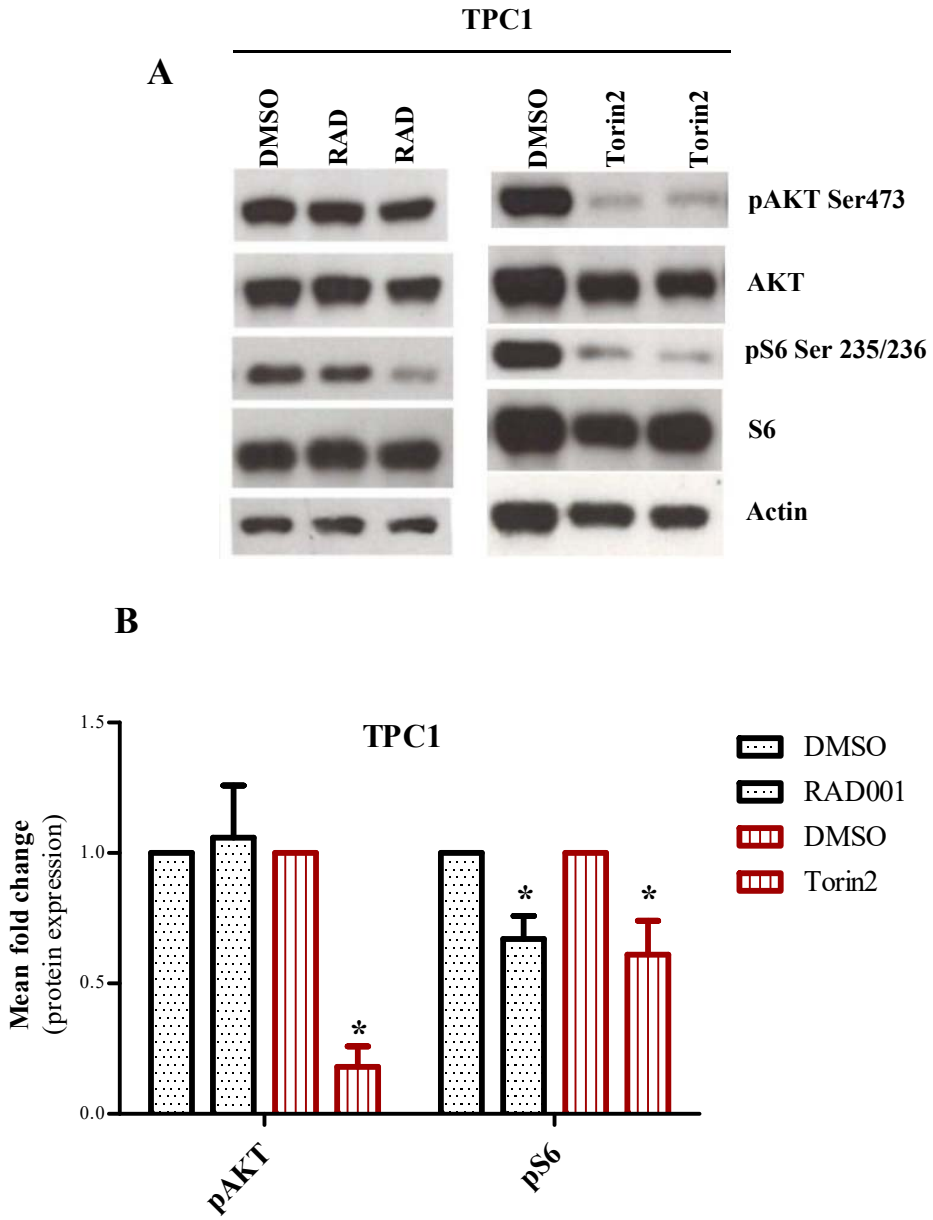


Figure 2. RAD001 and Torin2 effect on TPC1 cell line. **A**. Cells were treated with 20nM of RAD001 and 450nM of Torin2 during 72H. Western blot analysis of RAD001 and Torin2 effect on the activation status of mTORC1 and mTORC2 complexes was evaluated by pS6 Ser235/236 and pAKT Ser473 expression, respectively. Representative actin expression is shown. Protein level, in treated cells, was evaluated in duplicate. **B**. Mean fold change of protein expression observed in TPC1 cell line treated with 20nM of RAD001and 450nM of Torin2 in comparison to cells treated with DMSO. Phosphorylated proteins were normalized by the levels of their correspondent total proteins. Results are shown as mean expression value of three independent experiments ±SEM. *P<0.05 (unpaired Student's *t* test).

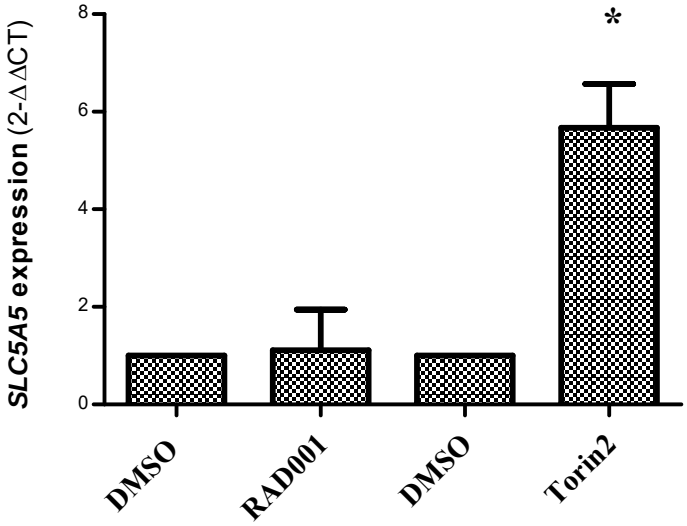


Figure 3. *SLC5A5* expression in TPC1 cell line after treatment with RAD001 (20nM) and Torin2 (450nM) during 72H. Mean fold change of *SLC5A5*mRNA expression observed in TPC1 cell line after treatments in comparison to cells treated with DMSO. RAD001 did not affect *SLC5A5* expression. Treatment with Torin2 caused a significant increase of ~6 fold of *SLC5A5* expression. Bars represent mean expression±SEM. * $P<0.05$. Results are shown as mean expression value of three independent experiments ±SEM.

4.7. Tables

Table 1. Epidemiologic, histological, and clinical data of the patients.

total and %	cPTC	FVPTC	other PTC variants
Gender			
F n=150	94 (82.5)	41 (87.2)	15 (71.4)
M n=32	20 (17.5)	6 (12.8)	6 (28.6)
Age			
<45 years n=94	62 (54.9)	21 (45.7)	11 (55.0)
≥45 years n=85	51 (45.1)	25 (54.3)	9 (45.0)
Tumor size			
<2cm n=64	39 (36.8)	17 (37.0)	8 (40.0)
≥2cm n=108	67 (63.2)	29 (63.0)	12 (60.0)
Tumor capsule			
Present n=83	42 (39.6)	32 (71.1)	9 (42.9)
Absent n=89	64 (60.4)	13 (28.9)	12 (57.1)
Tumor capsule invasion			
Yes n=64	35 (89.7)	22 (68.8)	7 (100)
No n=14	4 (10.3)	10 (31.3)	0 (0)
Extrathyroidal extension			
Yes n=73	50 (48.1)	12 (27.3)	11 (55.0)
No n=95	54 (51.9)	32 (72.7)	9 (45.0)
Multifocality			
Single n=104	58 (54.7)	32 (68.1)	14 (70.0)
Multiple n=69	48 (45.3)	15 (31.9)	6 (30.0)
Lymphocytic infiltrate			
Present n=108	77 (70.6)	19 (41.3)	12 (60.0)
Absent n=67	32 (29.4)	27 (58.7)	8 (40.0)
Vascular invasion			
Present n=59	42 (40.4)	10 (22.2)	7 (35.0)
Absent n=110	62 (59.6)	35 (77.8)	13 (65.0)
Lymph node metastases			
Present n=57	40 (43.0)	12 (34.3)	5 (29.4)
Absent n=88	53 (57.0)	23 (65.7)	12 (70.6)
Tumor margins			
Infiltrative n=78	57 (79.2)	13 (46.4)	8 (72.7)
Well defined n=33	15 (20.8)	15 (53.6)	3 (27.3)
Distant metastases			
Yes n=17	9 (11.8)	5 (17.9)	3 (30.0)
No n=97	67 (88.2)	23 (82.1)	7 (70.0)
One year disease free survival			
Yes n=64	41 (56.2)	19 (67.9)	4 (40.0)
No n=47	32 (43.8)	9 (32.1)	6 (60.0)
Disease free (at the end of follow up)			
Yes n=70	44 (59.5)	19 (67.9)	7 (70.0)
No n=42	30 (40.5)	9 (32.1)	3 (30.0)
Deaths			
Yes n=5	2 (2.6)	2 (7.1)	1 (9.1)
No n=110	74 (97.4)	26 (92.9)	10 (90.9)
BRAF			
WT n=106	56 (49.1)	37 (82.2)	13 (61.9)
V600E n=74	58 (50.9)	8 (17.8)	8 (38.1)
NRAS			
WT n=162	108 (99.1)	38 (90.5)	16 (80.0)
Mut n=9	1 (0.9)	4 (9.5)	4 (20.0)
TERTp			
WT n=152	95 (96.0)	40 (95.2)	17 (100.0)
Mut n=6	4 (4.0)	2 (4.8)	0 (0.0)
RET/PTC			
WT n=56	29 (78.4)	18 (94.7)	9 (90.0)
Rearrangment n=10	8 (21.6)	1 (5.3)	1 (10.0)
Staging			
I n=64	45 (64.3)	15 (60.0)	4 (50.0)
II n=6	3 (4.3)	3 (12.0)	0 (0.0)
III n=24	19 (27.1)	3 (12.0)	2 (25.0)
IV n=9	3 (4.3)	4 (16.0)	2 (25.0)

Table 2. Distribution of pAKT score throughout the series.

pAKT score	Frequency	%
0	90	49.5
1	18	9.9
2	15	8.2
3	6	3.3
4	8	4.4
6	14	7.7
8	11	6.0
9	6	3.3
12	14	7.7
Total	182	100

Table 3. Association between pAKT score and *BRAF* status.

		pAKT Score	*P* value
BRAF	WT (n=106)	2.2±3.3	0.04
	V600E (n=74)	3.4±4.4	

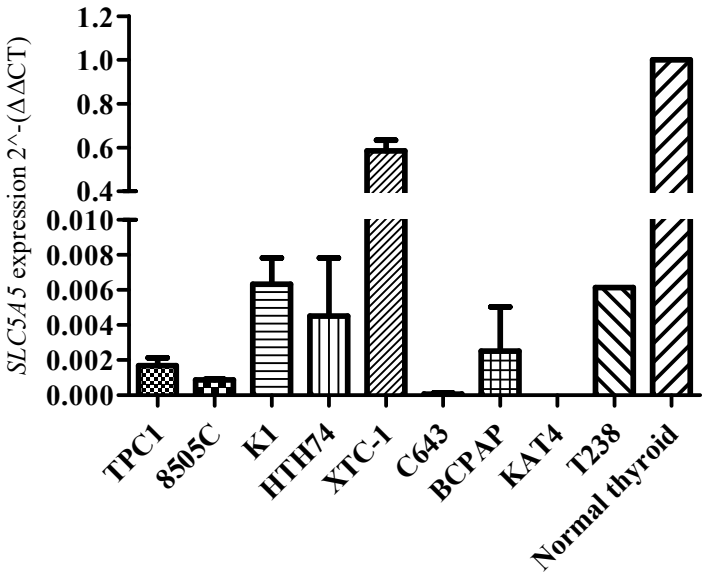
Table 4. Association between pAKT nuclear expression and distant metastases.

		Distant metastases		
		Yes	**No**	***P* value**
Nuclear expression	**Yes**	9 (81.82%)	19 (47.5%)	
	No	2 (18.18%)	21 (52.5%)	0.04
	Total	11	40	

Table 5. Effect of RAD001 and Torin2 on *SLC5A5* mRNA expression in TPC1 cell line.

	TPC1	
	***SLC5A5* expression**	***P* value**
RAD001		
DMSO 60H	1	
RAD001 20nM 60H	0.9±0.7	0.4
DMSO 72H	1	
RAD001 20nM 72H	1.1±0.8	0.5
Torin2		
DMSO 60H	1	
Torin 2 450nM 60H	1.3±0.6	0.4
DMSO 72H	1	
Torin 2 450nM 72H	5.7±0.9	**0.018**

4.8 Supplementary data



Supplementary figure 1. *SLC5A5* expression in a panel of thyroid carcinoma cell lines. Mean fold change of *SLC5A5* expression in comparison to a sample of normal adjacent human thyroid. Results were evaluated as mean expression in triplicate from two t biological replicates ±SEM.

Chapter 5. Paper 3 The usefulness of the study of sodium iodide symporter expression in thyroid primary tumors

This chapter is presently a manuscript submitted for publication with the same title.

The usefulness of the study of sodium iodide symporter expression in thyroid primary tumors

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Short title: NIS expression in primary thyroid carcinomas

Key words: Thyroid, cancer, NIS, mRNA, immunohistochemistry

Word count: 6536 words

Abstract

Objective: Thyroid cancer therapy is based on surgery followed by radioiodine treatment. The incorporation of radioiodine by cancer cells is mediated by sodium iodide symporter (NIS) (codified by *SLC5A5* gene), that is functional only when targeted to the cell membrane. We aimed to evaluate if NIS expression in thyroid primary tumors would be helpful in predicting tumor behavior, response to therapy and prognosis.

Design: NIS expression was addressed by qPCR and immunohistochemistry. In order to validate our data, we also studied *SLC5A5* expression on 378 primary papillary thyroid carcinomas from The Cancer Genome Atlas (TCGA) database.

Results: In our series, *SLC5A5* expression was significantly lower in carcinomas with vascular invasion and tendentially lower in those harboring *BRAF*V600E mutation and with extrathyroidal extension. Analysis of *SLC5A5* expression from TCGA database confirmed our results. Furthermore, it demonstrated that carcinomas larger than 2cm and with locoregional recurrences and/or distant metastases or harboring *RAS*, *BRAF*, and/ or *TERT* promoter (*TERT*p) mutations presented significantly less *SLC5A5* expression.

Regarding immunohistochemistry, 12/211 of the cases demonstrated NIS in the membrane of tumor cells, those cases showed variable outcomes concerning therapy success, prognosis, and all but one were wild type for *BRAF*, *NRAS* and *TERT*p mutations.

Conclusions: *SLC5A5* mRNA lower expression is associated with markers of aggressiveness and with key genetic alterations involving *BRAF*, *RAS* and *TERT*p. Mutations in these genes seem to decrease protein expression and its targeting to the cell membrane. *SLC5A5* mRNA expression is more informative than NIS immunohistochemical expression regarding tumor aggressiveness and prognosis.

5.1 Introduction

Sodium iodide symporter is a transmembrane glycoprotein (codified by the *SLC5A5* gene) expressed almost exclusively in the basolateral plasma membrane of thyroid follicular cells. It plays a central role in thyroid metabolism, mediating the active transport of iodine from the bloodstream into the follicular cells, the first step for thyroid hormones' synthesis. NIS plays an essential role in the treatment of differentiated thyroid carcinomas (DTC), which usually maintain NIS expression, allowing the recognition and the treatment of recurrences and metastases with radioactive iodine (RAI) [1]. Nonetheless, a significant subgroup of DTC patients with advanced disease loses NIS expression and becomes refractory to ^{131}I; some of these patients die within 3-5 years[2]. NIS expression has been widely studied in normal thyroid and tumor tissues, on one hand to verify if its downregulation could be the molecular cause for the decrease of RAI uptake and on the other hand to understand the impairing mechanisms of NIS expression and function. However, no clear answer emerged from the results obtained in the previous studies. Despite the central role of NIS in diagnosis, treatment and follow-up of thyroid cancer patients, reliable methods for ascertaining NIS expression and functionality in clinical samples are not available.

In the majority of the studies, *SLC5A5* mRNA levels are lower in thyroid carcinomas than in adenomas[3] and normal adjacent thyroid[4-6]; furthermore, *SLC5A5* expression presents some limitations in predicting NIS expression and functionality: whereas a negative or low mRNA level may lead to reduced protein expression, a positive or high mRNA expression does not always correspond to higher protein levels or higher functionality[6, 7].

These observations suggest that in thyroid carcinomas, besides transcription regulation, NIS expression appears to be modulated by post transcriptional events. Therefore, studies of NIS expression by immunohistochemistry (IHC)[1, 8-24], may be, theoretically, more informative since they "grab" NIS a step forward in its biological processing and allow the evaluation of the localization of NIS in the basolateral plasma membrane of follicular cells (the functional transporter).

According to the published data, NIS expression (evaluated by IHC) varies in different thyroid tissues. In normal thyroid, it is low and very heterogeneous; only a few follicular cells within some follicles express NIS in the basolateral plasma membrane[9, 13, 16, 20, 25], suggesting that, NIS expression is tightly regulated in thyroid gland. In carcinomas, when NIS is present, it is usually expressed in a higher number of cells than in normal tissue and the expression is mainly intracytoplasmic, poorly targeted to the basolateral plasma membrane[1, 10-13, 16, 20-22]. The increased intracytoplasmic NIS staining in thyroid tumors compared to normal tissue has been pointed out as a reason for the decreased RAI uptake in tumors, reflecting a mislocalization of NIS from the basolateral membrane, which would impair its activity[16]. This assumption has been questioned, because the real significance of intracytoplasmic NIS detected by immunostaining remains unclarified[20].

The molecular mechanisms responsible for the downregulation and/or not targeting to the basolateral membrane of NIS in thyroid tumors remain poorly understood, but some studies demonstrated that both mRNA and protein are differentially expressed according to the genetic background of the tumor. In fact, papillary thyroid carcinomas (PTCs) harboring the *BRAF*V600E mutation present lower *SLC5A5* mRNA and NIS protein expression as well as less targeting to the basolateral membrane compared to PTCs *BRAF*WT [18, 23, 26]. Less is known about the impact of other mutations on *SLC5A5* and NIS expression/targeting to the basolateral membrane.

Being NIS the central molecule for DTC treatment, it is logical to study if its expression in the primary tumor would be helpful in predicting therapy response as well as tumor behavior and prognosis. Some studies tried to understand if NIS immunohistochemical expression in thyroid primary tumors would be helpful in predicting ^{131}I uptake in recurrences and distant metastases. Although authors related that positive NIS immunostaining in primary tumors seemed to be predictive of positive recurrences and metastases on ^{131}I scans, some studies did not distinguish whether NIS was expressed in the cell basolateral membrane, and negative NIS staining did not predict ^{131}I scan-negative metastases [12, 14, 17]. To the best of our knowledge, there is only one study that addressed possible associations between NIS expression, evaluated by immunohistochemistry (IHC), and clinicopathological features and prognosis in a large series of

thyroid primary tumors[1], reporting a significantly lower NIS expression in older patients (≥45years) and also that NIS expression in the primary tumor was not useful as a prognostic marker.

So, in our opinion more retrospective studies in larger series of primary tumors are still necessary, in order to understand the role of NIS expression in therapy response, tumor behavior and prognosis, and also if other factors besides *BRAF*V600E mutation can contribute to NIS downregulation and/or misdirecting to the basolateral membrane. Furthermore, it is also important to understand the advantages and limitations of the analysis of *SLC5A5* and NIS expression and evaluate what is the better/more informative method to study NIS expression.

Having this in mind, we addressed *SLC5A5* expression by qPCR and NIS expression by IHC analysis, in a large series of primary thyroid carcinomas and looked for possible associations with some clinicopathological and molecular features, as well as to the response to RAI therapy and outcome. In order to validate our results of *SLC5A5* mRNA expression associations' with clinicopathological and molecular features and also to get new evidences we used the data available about *SLC5A5* in TCGA Research Network that completed an integrated genomic analysis of 496 PTCs using NGS and other pan-genomic technologies, together with detailed pathologic and clinical data[27].

5.2 Materials and methods

Patient samples

Our series was composed by 255 thyroid samples from 229 patients. Cases were collected from the files of the Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP, Porto, Portugal), corresponding to patients with thyroid tumors (n=229) operated and followed in two university hospitals. Samples from normal thyroid (n=25), and Graves' disease (n=1) were obtained from the contralateral lobe of the surgical specimens. Carcinomas series was composed by 193 PTCs (123 cases of classical PTC (cPTC), 47 cases of follicular variant of PTC (FVPTC) and 23 cases of other PTC variants), 23 follicular thyroid carcinomas (FTC) and 13 poorly differentiated thyroid carcinomas (PDTC). In 166 cases, there was only formalin-fixed paraffin-embedded (FFPE) representative tissue; in 45cases there were FFPE samples and correspondent frozen tissue (the tumors were divided at the time of surgery); and in 18 cases there was only frozen tissue available. Frozen material was collected at the time of surgery and conserved at-80ºC.The histology of all tumor samples was reviewed by three pathologists (CE, ER, MSS) according to the criteria of the World Health Organization[28]. Clinicopathological and molecular data of the 229 patients with carcinoma are summarized in Supplementary Table 1. In 141 cases, follow-up data was available. The number of ^{131}I treatments varied from 1 to 5 treatments (mean 1.9), and the cumulative total dose of RAI was between 30 and 1146 mCi (mean 251 mCi). All the procedures described in this study were approved by the respective ethical boards and are in accordance with national and institutional standards.

Patient follow up

Patients were treated and followed in accordance with the international protocols available at the time. Data regarding the number of radioiodine treatments and cumulative activity were retrieved from hospital records. Patients were considered as being disease-free at the end of follow-up if they had undetectable stimulated thyroglobulin (in the absence of thyroglobulin antibodies) and no evidence of the disease on radiographic or radionuclide imaging. The mean time of follow up was 8.0±6.7 years. For statistical analysis, we defined the category "additional

treatments", in which we included other treatment modalities in addition to radioiodine, including extra surgery, external beam irradiation, and treatment with tyrosine kinase inhibitors.

Dataset PTC in TCGA

There were 378 tumor cases for which there was information for the main driver somatic mutations (*RAS, BRAF* and *TERT*p), gender and *SLC5A5* expression. Of these, we eliminated 4 cases, for which the *SLC5A5* expression was above the 99 percentile, being outliers. A total of 353 of the cases had information about tumor size, 362 had information for extrathyroidal extension, 282 had information for lymph node metastases (at the time of diagnosis) and all 374 had information about new tumor event [lymph node metastases or local recurrence (grouped in locoregional recurrence) and distant metastases]. The *SLC5A5* expression was inferred from RNA-seq data and quantification reflects reads per kilobase per million mapped reads (RPKM). There were also 58 *SLC5A5* expression measures in adjacent tissue of the PTC cases, and two of them were not considered for further analyses as values were above the 99 percentile.

DNA extraction, PCR, and Sanger sequencing

DNA extraction from FFPE tissues was performed from 10μm sections after careful microdissection. DNA extraction was performed using Ultraprep tissue DNA kit (AHN Biotecnologie, Nordhausen, Germany) following the manufacturer's instructions. The genetic characterization of part of the tumors regarding *BRAF*, *NRAS,* and *TERT* promoter mutations (*TERT*p) had been reported previously; mutations were screened as previously described [29-31].

RNA extraction and reverse transcription

Total RNA was extracted from tumors and from contralateral normal adjacent thyroid, from which frozen samples were available (n= 84), using a Trizol commercial kit (Thermo Scientific/GIBCO Waltham, MA USA) according to the manufacturer's protocol. RNA was quantified by spectrophotometry, and its quality was checked by analysis of 260/280 nm and 260/230 nm ratios. For cDNA preparation, 1μg of total RNA was reverse-transcribed using the RevertAid first strand cDNA synthesis kit (Thermo Scientific/Fermentas, Waltham, MA, USA).

qReal Time PCR

Reverse transcription products were amplified for the *SLC5A5*gene and detected by a probe (IDT**:** Integrated DNA Technologies, Leuven, Belgium; no. HS.PT.56a.40789288), as previously described[32].

Immunohistochemistry

Immunohistochemistry was performed in normal thyroid and in 211 carcinomas. Briefly, deparaffinized and rehydrated sections were subjected to heat-induced antigen retrieval in 10 mM sodium citrate buffer (pH6.0). Endogenous peroxidase activity was blocked with 3% of hydrogen peroxide and nonspecific binding with Large Volume Ultra V Block reagent (Thermo Scientific/Lab Vision, Waltham, MA, USA). Sections were then incubated overnight at 4ºC with anti-NIS antibody (1:400) clone FP5A (Thermo Scientific/Lab Vision, Waltham, MA, USA) and in 24 carcinomas with anti-NIS pAb 795 IgG (20µg/ml) (kindly supplied by Dr. Ruby)[33]. Additionally, Tyramide Signal Amplification (TSA) Biotin System (Perkin-Elmer, Foster City, USA) was used for signal amplification in 44 carcinomas, according to manufacturer's instructions. The detection was performed with a labeled, streptavidin-biotin immunoperoxidase detection system (Thermo Scientific/Lab Vision Waltham, MA, USA) followed by 3,3'-diaminobenzidine (Dako, Glostrup, Denmark) and counterstained with hematoxylin. Graves' disease sample was used as a positive control and the negative control consisted in omission of the primary antibody.

Slides were evaluated by two observers and were analyzed according to the percentage of tumor stained cells, the intensity and the cellular localization of the staining. In order to compare our results to the literature, we considered cases with >5% of stained tumor cells (regardless of the cellular localization) as positive. Nevertheless, all our statistical analyses were performed considering two groups; cases that presented membrane staining in tumor cells and all the other cases. Photographs were acquired using Nikon DS-L1 camera in 100X and 400X magnifications.

Statistical analysis

Statistical analysis was performed using 21.0 SPSS Statistical Package (SPSS, Inc., 2003). Fisher's exact test, and independent-samples t-test were performed to correlate NIS expression with clinicopathological and molecular features. When parametric tests were not applicable we used alternative tests, specifically Mann-Whitney (independent samples). Wilcoxon (related samples) was used to compare *SLC5A5* expression between tumor samples and their adjacent normal counterparts. Kruskal-Wallis test was used to correlate *SLC5A5*expression (retrieved from TCGA and database) with clinicopathological and molecular features. Values of $P<0.05$ were considered statistically significant.

5.3 Results

***SLC5A5* mRNA expression**

SLC5A5 expression was significantly lower in carcinomas than in normal adjacent counterparts (Figure1). No significant difference was observed between the three different carcinoma histotypes (PTC, FTC and PDTC). Considering the analysis in DTC, *SLC5A5* expression was lower in males and in cases with vascular invasion ($P=0.003$ and $P=0.03$, respectively) (Table 1). *SLC5A5* expression in normal thyroid from males was not significantly different from that of females (data not shown). In addition, there was a tendency to lower *SLC5A5* levels in cases with extrathyroidal extension ($P=0.06$) and in PTCs harboring *BRAF*V600E mutation ($P=0.07$). When the statistical analysis was performed only in the PTC group all the significant associations described in the DTC group were maintained.

***SLC5A5* mRNA expression (TCGA database)**

The *SLC5A5* expression was around 200 times higher in normal tissue than in tumor tissue in both genders, but no differences in tumor and in adjacent tissue between genders were found (Figure 2 A and B). *SLC5A5* expression was significantly higher in smaller tumors ≤2cm (median= 5.85) compared to those with >2cm (median=2.51) ($P=0.028$; Figure 2 C). There was no statistical difference in *SLC5A5* expression in primary tumors with (median=3.0) or without (median=5.4) lymph node metastases at the time of diagnosis ($P=0.253$) (Figure 2 D).

The *SLC5A5* expression was reduced with the level of the extrathyroidal extension (median values: 5.4 for "none"; 2.8 for "minimal (T3)" and 0.9 for "moderate/advanced (T4a+b)"), reaching statistical significance for comparisons between "none" versus the "moderate/advanced (T4a+b)" class and "minimal (T3)" versus "moderate/advanced (T4a+b)" (P=0.018 and P=0.039, respectively Figure 2 E). We also observed a statistical significant decrease (from a median of 3.8 to 0.8; P=0.002) of the *SLC5A5* expression in cases with new tumor events (Figure 2 F), lumping together 12 cases of distant metastasis (6 lung; 1 lung+bone; 1 lymph node only; 1 lung+femur+neck+pleura+liver; 1 bone; 2 unknown) and 14 locoregional recurrences (10 lymph node only; 2 left thyroid; 1 lymph node + soft tissue; 1 unknown). Finally, *SLC5A5* expression was significantly higher in the absence (median=21.77) of the evaluated mutations: *RAS* (P=0.034), *TERT*p (P=0.0072) and *BRAF*V600E (P=3.1x10^{-8}). The PTCs that harbored only *TERT*p, only *BRAF* or simultaneous *TERT*p and *BRAF* mutations displayed significantly lower expression of *SLC5A5* than the double WT tumors. The group with *RAS* mutations displayed the second highest expression value (median=7.50), reaching statistical significance when compared with the groups including *BRAF* mutation only and *BRAF*+*TERT*p mutations [median=2.27 in *BRAF* (P=0.042); median=1.89 in *TERT*+*BRAF* (P=0.027)] (Figure 2 G).

NIS expression

In normal thyroid tissues, NIS immunohistochemical expression was mainly localized in the basolateral plasma membrane of follicular cells. NIS positivity was detected in a few foci of isolated follicles throughout the tissue and within the positive follicles the majority of the cells were positive. Positivity was more frequently detected in small follicles composed by cuboidal and columnar cells and rarely detected in large follicles limited by flattened cells (Figure 3A). In Graves' disease, NIS was widely expressed and present in the basolateral plasma membrane of the great majority of follicular cells (Figure 3B). In carcinomas, NIS staining was observed in 71.6% of the cases (74.8% of cPTCs, 69.8% of FVPTCs, 80.9% of other PTC variants, 55% of FTC and 67% of PDTC). Its location was predominantly in the cytoplasm (124/211) (Figure 3C)

in the cytoplasm and nucleus (15/211) and finally only 12/211 of the cases presented NIS in the basolateral plasma membrane of tumor cells (Figure 3D).

Since we observed a low percentage of carcinomas with NIS staining in the basolateral membrane, we hypothesized that our IHC approach was not being sensitive enough to detect small amounts of NIS. In order to clarify this issue, we used two strategies: a TSA signal amplification method and the use of another NIS antibody characterized by a different specificity compared to the commercial antibody[33].

The TSA signal amplification method was applied in a subset of 44 carcinomas with different staining patterns (16 with cytoplasmic staining in the tumor and membrane staining in adjacent thyroid; 3 with membrane staining in the tumor; 5 negative both in the in tumor and the adjacent thyroid and, finally, 20 with only cytoplasmic staining in tumor and adjacent thyroid). When we compared the slides with and without amplification, we verified that only the membrane staining remained and appeared more intense with the amplification. In these cases, the staining involved almost always the same foci of cells that already presented membrane staining (Figure 2E, F, G and H) i.e. it did not stained additional cells. The intra cytoplasmic staining vanished both in cancer and in normal tissues. Furthermore, we performed IHC using a homemade antibody for human NIS, pAb 795 against a peptide corresponding to the C-terminal sequence of hNIS pAb 795[33] in 24 carcinomas (12 cPTC, 4 FVPTC, 2 micro PTC, 2 tall cell PTC, 2 FTC and 2 PDTC). The results were similar to those obtained with clone FP5A (Thermo Scientific/Lab Vision, Waltham, MA, USA) (Supplementary Table 2).

Since some doubts remained about the specificity of the cytoplasmic staining, and also because NIS is only active when present in the basolateral membrane of the cells, we performed statistical analysis dividing our series in two groups: with (positive) and without (negative) membrane staining.

We did not find any significant association between NIS expression in the membrane and age, tumor size, tumor capsule, multifocality, lymphocytic infiltration, vascular invasion, lymph node metastases, tumor margins, distant metastases, staging, *BRAF*, *NRAS* and *TERT*p status, additional treatments, disease-free status at one year, disease-free status at the end of follow-up

or disease-specific survival in the DTC group. When we analyzed NIS expression between WT PTCs and those harboring any of the studied mutations we verified that NIS positive expression was significantly more frequent in WT PTCs (Table 2). The number of RAI therapies, as well as the cumulative dose of RAI, did not differ significantly between patients with or without NIS expression in the basolateral membrane of primary tumor's cells.

The throughout analysis of the few cases with membrane staining (n=12) revealed that all but one carcinoma were wild type for the studied mutations (*NRAS*+ *BRAF* or *TERT*p). These cases presented variable outcomes i.e. presence of distant metastases, number of RAI therapies, cumulative dose of RAI, the need of additional treatments, disease free status and death (disease caused), that are apparently unrelated with the presence of NIS membrane expression (Table 3).

5.4 Discussion

In this work, we tried to clarify the impact of NIS expression (mRNA and protein) on thyroid tumors' aggressiveness and therapy success and, as a result of the above, the putative prognostic significance of *SLC5A5* mRNA and NIS protein expression. Moreover, we also addressed the impact of the genetic background of the tumor on *SLC5A5* and NIS expression as well as its targeting to the basolateral cell membrane.

We found that *SLC5A5* expression was always lower in tumors than in normal adjacent counterparts as reported by others groups[5, 6, 34]. We observed a significantly lower *SLC5A5* expression in male gender patients, and in cases with vascular invasion, as well as a tendency to lower *SLC5A5* expression in cases with extrathyroidal extension, but no differences were found in cases with and without lymph node metastases (Table 1).When we compare the results from our data to those from TCGA data, we confirmed that tumors express significantly less *SLC5A5* compared to normal adjacent tissue, that *SLC5A5* was not differently expressed in the presence or absence of lymph node metastases (at the time of diagnosis) and a significant lower *SLC5A5* expression in tumors with extrathyroidal extension (moderate/advanced) compared to those without extrathyroidal extension (Figure 2D). However, the differential expression of *SLC5A5*

between genders was not confirmed (Figure 2 A, B). Unfortunately, in TCGA database there was no information about vascular invasion, so we could not validate this result in this large series.

The significantly lower *SLC5A5* expression in cases presenting vascular invasion and extrathyroidal extension suggests that a decreased *SLC5A5* expression may be associated to an aggressive tumor behavior and thus may help to characterize patients at risk for poor therapy response. Further analysis of TCGA data demonstrated that *SLC5A5* expression is significantly lower in cases that had locoregional recurrences and/or distant metastases (Figure 2 E). Given the high prognostic impact of recurrences and distant metastases[35], these results suggest that a lower expression of *SLC5A5* in thyroid primary tumor seems to be associated with features of higher aggressiveness of the primary tumor and also with a worse prognosis and with poor response to therapy. Two groups reported that *SLC5A5* was significantly less expressed in DTCs larger than 2cm and PTCs larger than 1cm (in comparison to ≤ 2cm and > 1cm, respectively)[1, 36], TCGA results corroborated the literature by showing that smaller PTCs (≤ 2cm) expressed significantly less *SLC5A5* compare to those with > 2cm. In our study we did not include microcarcinomas, so the group of tumors with ≤2 cm was very small, in any way we tested for a possible correlation between tumor size (as a continuous variable) and *SLC5A5* expression and did not find any significant correlation, the same analysis was performed in TCGA data (in the group of PTCs >2cm) and there was no significantly correlation either (data not shown).

Previous studies reported a lower *SLC5A5* expression in cases harboring *BRAF*V600E and there is experimental evidence showing that *BRAF*V600E can impair *SLC5A5* expression[1, 18, 26, 36], nevertheless the impact of other relevant mutations found in thyroid tumors on *SLC5A5* expression remained unknown. In our series, *SLC5A5* expression was lower but did not reach statistical significance in the *BRAF*V600E PTC compared to that of *BRAF* wild type group. The lack of significance in our series may be due to differences in size and composition of the series, since the above mentioned studies addressing *SLC5A5* expression and *BRAF*V600E[1, 18, 26, 36] used larger series of PTC.

When we compared *SLC5A5* expression (retrieved from TCGA database) between PTCs harboring different mutations (*BRAF*V600E, *TERT*p and *RAS*) and WT PTCs we observed that

independently of the mutation, *SLC5A5* expression was always significantly lower compared to WT PTCs. Moreover, we also observed that *RAS* mutation was the one with lower impact on *SLC5A5* expression. PTCs with *RAS* mutation displayed significantly higher levels of *SLC5A5* compared to *BRAF*V600E and *BRAF*+TERp mutated PTCs. In fact, it has been previously reported a distinct profile of expression of genes involved in thyroid hormone biosynthesis (being *SLC5A5* one of these genes) between *BRAF*V600E and *RAS*-driven PTCs, with *RAS*-like PTCs having relatively high thyroid differentiation score[27].

Our results on the immunohistochemical NIS expression in normal thyroid and Graves' disease (an autoimmune condition known to express high levels of NIS)[37]were in accordance to data previously reported [9, 11, 13, 16, 20], i.e., focal membrane expression of NIS in normal thyroid gland and a widespread, strong membrane NIS expression in Graves' disease. The great difference observed in NIS expression between normal thyroid and in Graves' disease may be considered as an example of how TSH is able to regulate NIS expression and the targeting to the membrane. Regarding carcinomas, we observed that the majority (71.6%) displayed NIS immunostaining, which is in accordance to the literature [12, 13, 16-22] (Table 4), but only a minority presented NIS in the basolateral membrane of tumor cells (5.7%). If one compares the percentage of cases with NIS plasma membrane staining, there are large differences between studies (Table 4). Such differences may be due to the variable size of the series and also to differences in the antibodies used to perform the IHC (almost every study uses its own antibody, Table 4). In order to be sure that we were not missing any signal, we performed the IHC for NIS with TSA signal amplification in a subset of carcinomas with different immunostaining patterns and observed a complete vanish of intracytoplasmic staining and an amplification of the membrane staining. These results, like those from Peyrottes *et al*[20] rise some questions about the real significance of NIS intracytoplasmic staining, so we decided to perform our analysis considering positive only the cases with membrane staining.

The presence of NIS in the membrane of thyroid primary carcinomas did not associate with clinicopathological features, response to therapy or prognosis (Table 3). If we look to the treatment of thyroid carcinoma (surgery followed by RAI ablation), only the remnants, metastases

and eventually the recurrences are subjected to RAI. Prior to RAI ablation patients are subjected to TSH stimulation, either by withdrawal of thyroid hormones or by the administration of recombinant TSH[2]. Since TSH has a major role in NIS expression and targeting to the membrane[38], we can hypothesize that levels of membrane NIS in stimulated recurrences and metastases may be different from those in non-stimulated primary tumors because they may reflect two different biological conditions. This probably may help to explain why NIS expression in the primary tumor does not predict RAI therapy success and/or prognosis.

Another interesting finding of our study was the observation that the cases with NIS membrane staining were predominantly wild type for the analyzed mutations (*NRAS*, *BRAF* and *TERT*p) (Table 3). Although this membrane expression was not associated with any particular outcome (clinicopathological features or prognosis), it is tempting to advance that the genetic background of tumors influence NIS targeting to the membrane. There are *in vitro* evidences that *BRAF*V600E mutation affects NIS targeting to the membrane [23], but the impact of the other mutations (*NRAS* and *TERT*p) remains unknown.

In summary, *SLC5A5* mRNA expression was significantly lower in mutated PTCs and the absence of *BRAF* and *NRAS* mutations in every carcinoma displaying NIS membrane staining at immunohistochemistry supports the assumption that the genetic background of tumors may be of major importance to *SLC5A5* expression as well as to NIS targeting to the membrane. Moreover a lower *SLC5A5* mRNA expression was associated with tumor aggressiveness and worse prognosis. On the other hand, NIS immunohistochemical expression did not predict tumor behavior, therapy response or outcome. Thus, the study of *SLC5A5* mRNA expression is much more informative compared to NIS expression evaluated by IHC.

Declaration of interests

The authors have nothing to declare.

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5.6 Figures/Figure legends

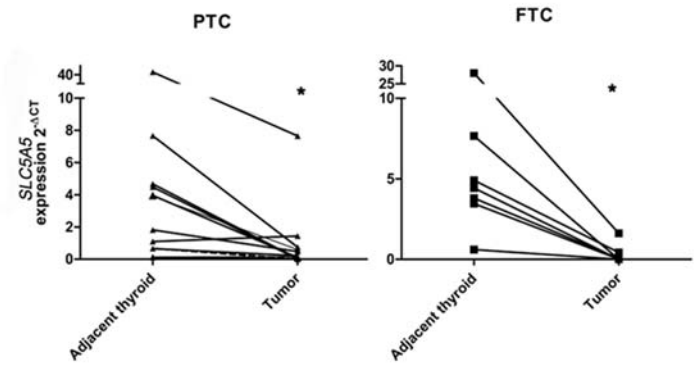
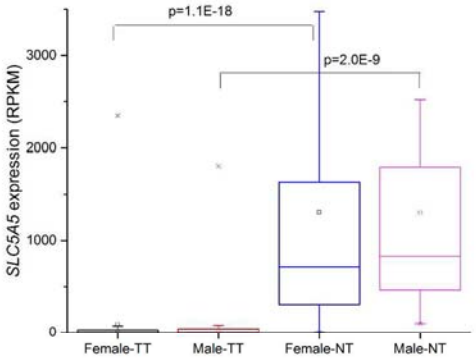
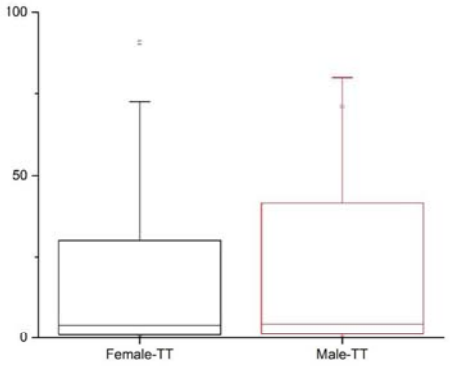
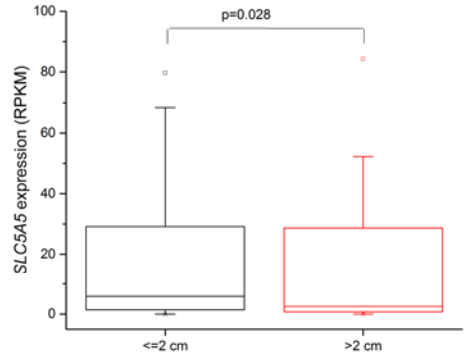
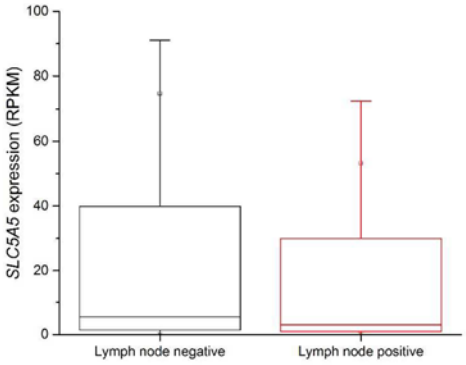


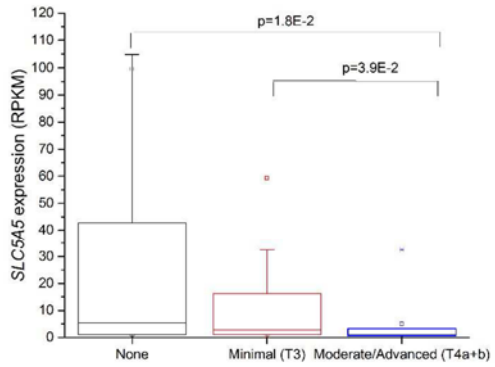
Figure 1. *SLC5A5* expression in thyroid carcinomas and paired normal adjacent counterparts.

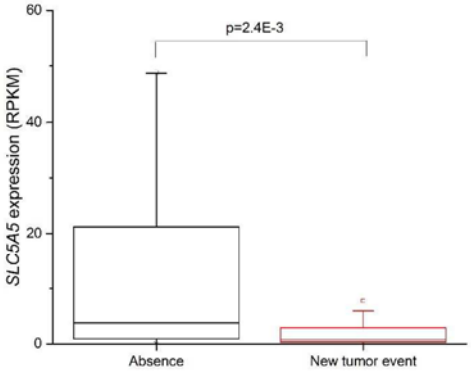

p=1.1E-18
p=2.0E-9
SLC5A5 expression (RPKM)
3000
2000
1000
0
Female-TT
Male-TT
Female-NT
Male-NT


100
50
0
Female-TT
Male-TT


p=0.028
SLC5A5 expression (RPKM)
100
80
60
40
20
0
<=2 cm
>2 cm


SLC5A5 expression (RPKM)
100
80
60
40
20
0
Lymph node negative
Lymph node positive


p=1.8E-2
p=3.9E-2
SLC5A5 expression (RPKM)
120
110
100
90
80
70
60
50
40
30
20
10
0
None
Minimal (T3)
Moderate/Advanced (T4a+b)


p=2.4E-3
SLC5A5 expression (RPKM)
60
40
20
0
Absence
New tumor event

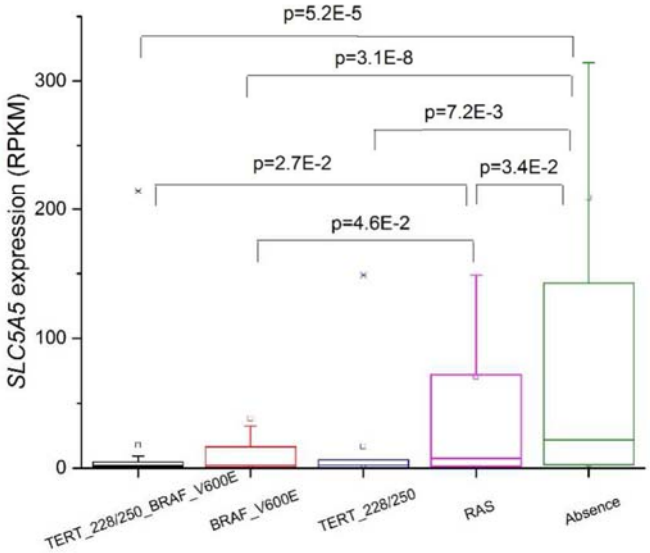

p=5.2E-5
p=3.1E-8
p=7.2E-3
p=2.7E-2
p=3.4E-2
p=4.6E-2
SLC5A5 expression (RPKM)
300
200
100
0
TERT_228/250_BRAF_V600E
BRAF_V600E
TERT_228/250
RAS
Absence

Figure 2. *SLC5A5* expression in primary PTCs (RPKM), data retrieved from TCGA database. Comparative analysis of *SLC5A5* expression **A**) between genders in tumor (TT) and normal tissue (NT); **B)** between genders only in tumor tissue (TT); **C)** in tumors with ≤2cm and >2 cm **D)** in cases with or without lymph node metastases at the time of diagnosis; **E)** in cases without, with minimal (T3) and with moderate/advanced extrathyroidal extension; **F)** in cases with and without recurrence; and **G)** between cases with different genetic backgrounds (WT, *RAS* mutation, *TERT*p mutation, *BRAF* mutation, *BRAF+TERT*p mutation). The boxes represent the interquartile range; the whiskers are the 5% and 95% quartiles; the small open boxes are the mean values; and the lines are the median values. Significant values for the Kruskal-Wallis test are indicated.

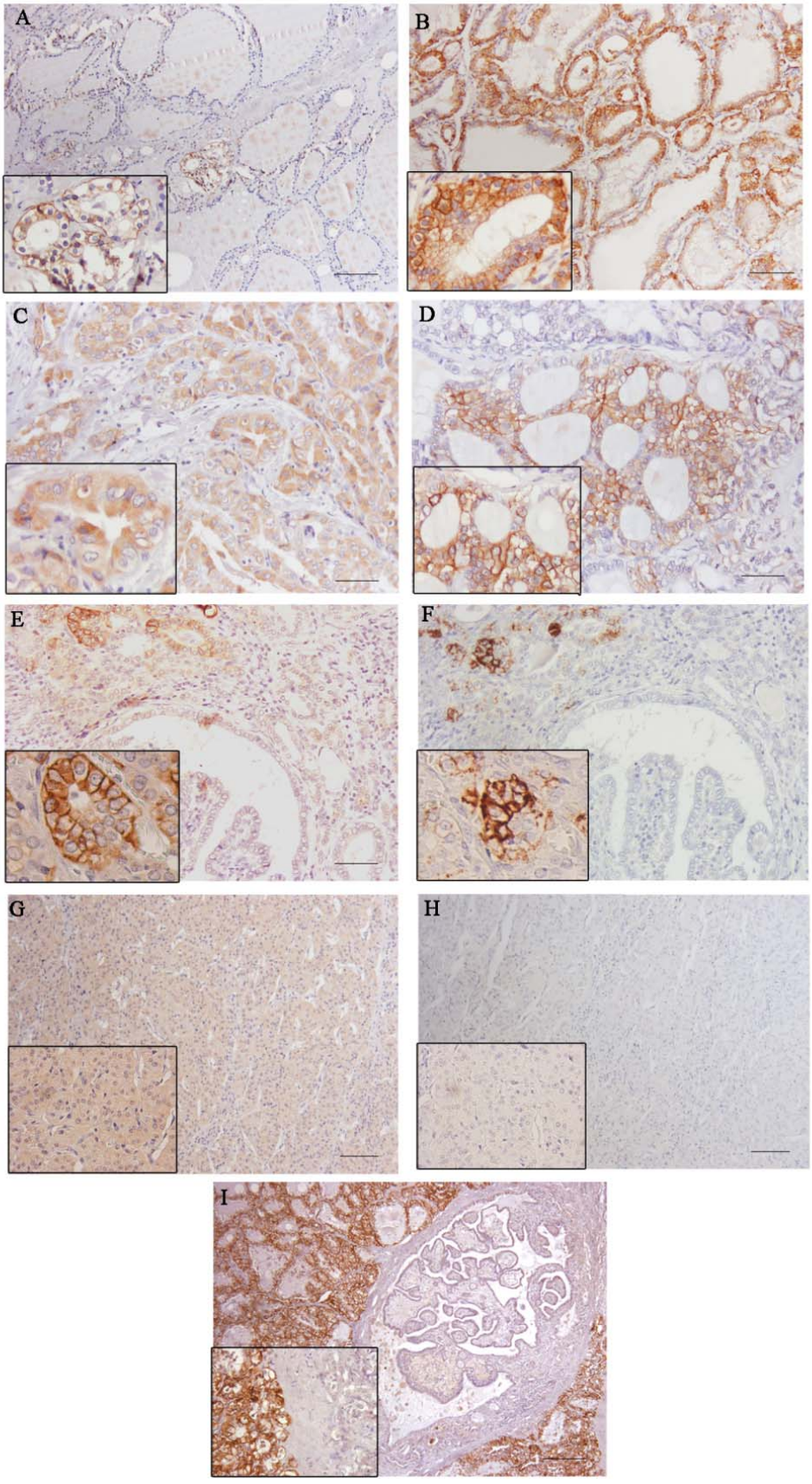

A
B
C
D
E
F
G
H
I

Figure 3. NIS immunoexpression in different thyroid tissues. **A)** normal thyroid; **B)** Graves' disease; **C)** cytoplasmic staining in an oncocytic PTC**; D)** membrane staining in a FVPTC; **E and F)** NIS immunoexpression in a FVPTC without and with TSA amplification signal, respectively; **G and H)** NIS immunoexpression in a FTC without and with TSA amplification signal, respectively; **I)** negative staining in cPTC with strong membrane staining in the surrounding Graves' disease. In E, F, G and H, notice the loss of cytoplasmic staining and the amplification of the membrane staining (E, F) after the use of TSA amplification system. Bar 100μm.

5.7 Tables

Table 1. Associations between *SLC5A5* expression with clinicopathological and molecular features in DTCs.

		***SLC5A5* expression**	***P* value**
Gender	F (n=47)	1.2±2.2	
	M (n=12)	0.2±0.2	**0.003**
Age	<45 years (n=30)	1.0±1.5	
	≥45 years (n=29)	1.1±2.4	0.8
Tumor capsule	Present (n=27)	1.1±1.6	
	Absent (n=30)	0.7±1.6	0.4
Tumor capsule invasion	Yes (n=17)	0.9±1.6	
	No (n=11)	1.4±1.4	0.4
Extrathyroidal extension	Yes (n=17)	0.5±1.1	
	No (n=37)	1,4±2.4	0.06
Lymphocytic infiltration	Present (n=19)	0.9±1.9	
	Absent (n=37)	1.2±2.2	0.7
Vascular invasion	Present (n=28)	0.4±0.8	
	Absent (n=29)	1.5±2.6	**0.03**
Lymph node metastases	Present (n=13)	0.5±0.8	
	Absent (n=18)	0.4±0.7	0.8
BRAF*	WT n= (27)	1.6±2.7	
	V600E (n=20)	0.5±1.0	0,07
NRAS	WT (n=54)	1.0±2.0	
	Mut (n=6)	1.3±1.7	0.7

***** PTC only

Table 2. Associations between NIS expression and clinicopathological and molecular features in PTCs.

	NIS immunoexpression		
	Negative	Positive	*P* value
Genetic background n=118			
WT	45 (41.3%)	8 (88.9%)	
Mutated#	64 (58.7%)	1 (11.1%)	0.011

(*BRAF*, *NRAS* or *TERT*p mutations)

Table 3. Clinicopathological and molecular data of cases presenting NIS membrane staining.

	Diagnosis	***BRAF***	***NRAS***	***TERT*p**	**Lymph node metastases**	**Distant metastases**	**Number of ^{131}I therapies**	**Cumulative dose (mCi)**	**Aditional treatments**	**One year DFS***	**DFS*#**	**Deaths**
Case 1	cPTC	WT	WT	WT	no	bone	3	457.5	no	no	no	no
Case 2	cPTC	WT	WT	WT	no	no	1	63	no	yes	yes	no
Case 3	cPTC	WT	WT	WT	yes	no	3	459	2 surgeries	no	no	no
Case 4	FVPTC	WT	WT	WT	yes	no	1	37	no	yes	yes	no
Case 5	FVPTC	WT	WT	WT	no	no	2	382	no	no	no	no
Case 6	PDTC	WT	WT	WT	no	lung+bone	5	798	2 surgeries	yes	no	no
Case 7	cPTC	WT	WT	124>A	yes	no	4	527	U/I	no	no	no
Case 8	sclPTC	WT	WT	WT	yes	no	3	400	U/I	no	no	no
Case 9	FTC	WT	WT	WT	no	no	1	102	U/I	U/I	yes	no
Case 10	cPTC	WT	WT	WT	yes	U/I	U/I	U/I	U/I	U/I	U/I	U/I
Case 11	FVPTC	WT	WT	WT	U/I	U/I	U/I	U/I	U/I	U/I	U/I	U/I
Case 12	cPTC	WT	WT	WT	yes	U/I	U/I	U/I	U/I	U/I	U/I	U/I

*DFS disease free survival. # At the end of follow up. U/I unavailable information. sclPTC sclerosing variant of PTC.

Table 4. Bibliographic revision and present results of NIS protein evaluation by IHC in thyroid carcinomas.

	Nº of carcinomas	Anti-NIS antibodies used in the study	Negative cases	Positive cases*	Cases with membrane staining**
Jhiang et al 1998	4 DTCs	Produced by authors	Descriptive studies		
Caillou et al., 1998)	14 DTCs	Produced by authors			
Saito et al., 1998	12 PTCs	Produced by authors			
Castro et al., 1999	9 DTCs	Produced by authors			
Castro et al., 2001	60 DTCs	Clone FP-13	26.7%	73.3%	N/A
Dohan et al., 2001	57 (53DTCs. 2 ATC; 2 MTC)	Produced by authors	29.8%	70.2%	15.8%
Min et al., 2001	67 DTCs	Donated by Dr. SM Jhiang of Ohio State University, USA	67.2%	32.8%	N/A
Wapnir et al., 2003	90 (87 DTCs; 3 ATC)	Produced by authors	22.5%	77.5%	some
Riesco Eizaguire et al., 2006	67 PTCs	Pohlenz et al., 2000	N/A		some
Lee etal., 2007	17 PTCs	Clone Ab-1	0%	100%	58.8%
Romei et al. 2008	40 PTCs	Brahms Diagnostica GmbH, Berlin, Germany	0%	100%	52.5%
Jung et al., 2009	29 (25 DTCs; 4 ATC)	Clone FP5A	37.5%	62.5%	N/A
Peyrottes et al., 2009	47 (42 DTCs; 5MTC)	Clones 39S, Ab-1 and FP5A	49%	51%	0%
Riesco Eizaguire et al., 2009	50 PTCs	Tazebay et al., 2000	N/A		8%
Wang et al. 2011	32 DTCs	Zhongshan Goldbridge Biotechnology, Beijin China	0%	100%	18.8%
Morari et al. 2011#	265 DTCs	Clone FP5A	88%	12%	12%
Wei et al., 2013	370 PTCs	Clone SPM186	32.7%	67.3%	0.8%
Tavares et al., present study	211 (199 DTCs; 12PDTCs)	Clone FP5A	28.4%	71.6%	5.7%

*Percentage of positive cases (independently of the cellular location). **Percentage of cases with NIS membrane staining with or without simultaneous cytoplasmic staining. # This specific study only considered positive cases with membrane staining. N/A not addressed. ATC Anaplastic thyroid carcinoma. MTC Medullary thyroid carcinoma. PDTC Poorly differentiated thyroid carcinoma.

5.8. Supplementary data

Supplementary Table 1. Clinicopathological data of the 229 patients with carcinomas included in the study.

	Total and (%)	PTC	FTC	PDTC
Age (n)	226	191	22	13
≥45 years	115(50.9)	91(47.6)	13(59.0)	11(84.6)
Gender (n)	228	193	22	13
Male	50(21.9)	39(20.2)	6(27.3)	5(38.5)
Tumor size (n)	212	181	20	11
≥2cm	161(75.9)	130(71.8)	20(100)	11(100)
Tumor capsule (n)	203	175	18	10
Present	108(53.2)	84(48.0)	18(100)	6(60)
Tumor capsule invasion (n)	109	86	17	6
Yes	90(82.6)	67(77.9)	17(100)	6(100)
Extrathyroidal extension (n)	196	170	18	8
Present	78(39.8)	76(44.7)	0(0)	2(25)
Multifocality (n)	198	174	15	9
Multifocal	86(43.4)	73(42.0)	9(60.0)	4(44.4)
Lymphocytic infiltrate (n)	197	170	18	9
Present	111(56.3)	102(60.0)	6(33.3)	3(33.3)
Lymph node metastases (n)	186	160	16	10
Present	93(50)	89(55.6)	0(0)	4(40.0)
Vascular invasion (n)	198	172	18	8
Present	80(40.4)	64(37.2)	9(50.0)	7(87.5)
Tumor margins (n)	124	114	5	5
Infiltrative	83(66.9)	81(71.1)	1(20.0)	1(20.0)
Distant metastases (n)	145	128	9	8
Present	33(22.8)	22(17.2)	5(55.6)	6(75.0)
Staging (AJCC) (n)	118	107	5	6
I	68(57.6)	65(60.8)	1(20.0)	2(33.3)
II	10(8.5)	7(6.5)	2(40.0)	1(16.7)
III	29(24.6)	26(24.3)	1(20.0)	2(33.3)
IV	11(9.3)	9(8.4)	1(20.0)	1(16.7)
One year disease free (n)	136	122	7	7
No	64(47.1)	56(45.9)	6(85.7)	2(28.6)
Disease-free (end of follow up) (n)	141	125	8	8
No	61(43.3)	51(40.8)	6(75.0)	4(50.0)
Deaths (n)	146	129	9	8
Yes	9(6.2)	5(3.9)	1(11.1)	3(37.5)
***BRAF* (n)**	226	191	22	13
V600E	82(36.3)	79(41.4)	0(0)	3(23.1)
***NRAS* (n)**	207	176	19	12
Mutation	12(5.8)	8(4.5)	3(15.8)	1(8.3)
***TERT*p (n)**	201	168	20	13
Mutation	19(9.5)	11(6.5)	5(25.0)	3(23.1)

Supplementary Table 2. Description of the immunolocalization of NIS in the cases stained with the two different anti-NIS antibodies: FP5A and pab795.

	FP5A	pab795
	Cellular location	**Cellular location**
Case 1	Negative	Cytoplasmic
Case 2	Cytoplasmic	Cytoplasmic
Case 3	Cytoplasmic	Cytoplasmic
Case 4	Membrane+Cytoplasmic	Membrane+Cytoplasmic
Case 5	Cytoplasmic	Cytoplasmic
Case 6	Cytoplasmic	Cytoplasmic
Case 7	Cytoplasmic	Cytoplasmic
Case 8	Cytoplasmic	Cytoplasmic
Case 9	Cytoplasmic	Cytoplasmic
Case 10	Cytoplasmic	Cytoplasmic
Case 11	Cytoplasmic	Cytoplasmic
Case 12	Cytoplasmic	Cytoplasmic
Case 13	Cytoplasmic	Cytoplasmic
Case 14	Cytoplasmic	Cytoplasmic
Case 15	Cytoplasmic	Cytoplasmic
Case 16	Cytoplasmic	Cytoplasmic
Case 17	Cytoplasmic	Cytoplasmic
Case 18	Cytoplasmic	Cytoplasmic
Case 19	Cytoplasmic	Cytoplasmic
Case 20	Cytoplasmic	Cytoplasmic
Case 21	Negative	Cytoplasmic
Case 22	Cytoplasmic	Cytoplasmic
Case 23	Cytoplasmic	Cytoplasmic
Case 24	Cytoplasmic	Cytoplasmic

Chapter 6. General discussion and concluding remarks

Differentiated thyroid carcinomas carry in general a very good prognosis, with high rates of disease free survival[149]. Unfortunately, a subgroup of DTCs' patient (4-23%) will develop distant metastases, worsening their prognosis[148]. The situation gets worse when recurrences and /or distant metastases patients lose the ability to uptake RAI, a situation that occurs in approximately 26-60% of the patients with recurrent disease[149]. This subgroup of DTC patients represents a challenge in TC field because there are not robust predictors that could help to identify such cases at the time of diagnosis. There is a great need of new predictors of aggressiveness and therapy response in TC, to avoid unnecessary overtreatment and, at the same time, to maintain an adequate disease management and surveillance.

mTOR pathway is overactivated in a great variety of human neoplasms, being sometimes associated with characteristics of aggressiveness and worse prognosis[155]. In TC, mTOR pathway is also overactivated; the effectors of the two mTOR complexes pS6 (mTORC1) and pAKT (mTORC2) are overexpressed in tumors compared to normal adjacent tissue[210], but the consequences of such overexpression in terms of tumor clinical behavior, prognosis and response to therapy remain unknown. Moreover, mTOR pathway may be involved in NIS expression regulation; *in vitro* studies demonstrated that inhibition of mTORC1 complex caused an increase of NIS expression and function[250, 251]. Having these in mind, our first and second objectives were to evaluate the impact of mTOR pathway status in the clinical behavior and prognosis of PTCs, and whether or not mTOR pathway plays a role on NIS expression/function.

Our results showed that pmTOR expression was associated with absence of tumor capsule, presence of distant metastases, persistence of disease, and *RAS* mutation, all characteristics of higher recurrence rates/worse prognosis[61, 88], thus appearing as a marker of aggressiveness in PTCs. Additionally, pmTOR positive expression was a predictive factor for distant metastization in univariate analysis, together with male gender and vascular invasion. When all of these

parameters were included in a multivariate analysis, pmTOR positive expression revealed to be an independent predictor of distant metastization.

Regarding the effectors of pmTOR, we observed that pS6 expression was associated with less aggressive pathological features, such as presence of tumor capsule, absence of extrathyroidal extension, well defined tumor margins and *BRAF* wild type status, while pAKT expression was significantly higher in PTCs harboring *BRAF*V600E mutation, and nuclear expression of pAKT was associated with the presence of distant metastases. We did not find a significant correlation between the expression of pmTOR and pS6 expression but the expressions of pmTOR and pAKT were significantly and positively correlated - PTCs with higher levels of pmTOR presented higher levels of pAKT. The positive and significant correlation between pmTOR and pAKT and the significant association between pAKT expression and distant metastization, also described for pmTOR[253], indicates that, in PTC, mTORC2 complex may be more relevant in terms of guarded prognosis. mTOR activation is leading to the activation of mTORC2 complex and the nuclear translocation of its downstream effector pAKT may play a major role in distant metastization (Figure 4).

mTOR pathway association with essential steps in the metastatic cascade was already observed in other tumor models. The impairment of mTORC1 and/or mTORC2 complexes assembly inhibited the capacity of cells to migrate and invade in some human carcinoma cell lines[198, 199, 202]. Even though both mTOR complexes are involved with features of cell motility and metastization, mTORC2 is more often correlated with such features[198, 199]. Preferential formation of the mTORC2 complex in tumor models and human malignancies was previously observed, and it is usually associated with metastization. In models of breast cancer and renal cell carcinoma, only mTORC2 (and not mTORC1) inhibition was able to impair cell motility and metastization[198, 199].In gastric, colorectal and breast cancer, pAKT expression (and not pS6 expression) was associated with metastization[196, 254, 255].

pAKT expression has also been associated with metastization in animal models of TC[216, 217]. Our results point out the activation of pAKT as an important step for PTC distant metastization. We observed that pAKTSer473 expression was associated with distant metastization only when we considered its nuclear expression. In fact, it seems that pAKTSer473 nuclear translocation is of major importance for migration and distant metastization of DTC. Vasko et al.,[209]demonstrated that pAKT Ser473 was expressed in the cytoplasm of PTC throughout the tumor, but the immunostaining was more intense and localized in the nucleus of cells located in the invasive regions. We also observed that when pAKTSer473 staining was more concentrated in the invasive front of the tumor, it was preferentially located in the nucleus. Moreover, in an animal model of TC, pAKT Ser473 was localized primarily in the nucleus of cells from metastatic lesions, while in the primary tumors it was located in the cytoplasm and in the nucleus, suggesting that pAKT nuclear distribution may be relevant both for the initiation and the sustaining of the metastatic process[217].

The lack of correlation between pmTOR and pS6 expression, as well as the distinct behavior of both markers of the same pathway is intriguing. One may speculate that pS6 may be regulated by other factors rather than pmTOR. On record there are reports pointing to other mechanisms that may cause S6 phosphorylation alternatively to mTOR, such as the casein kinase 1 (CK1), a ubiquitously expressed protein, involved in many biological processes including DNA repair, cell cycle control, and circadian rhythm entrainment [256], and RSK that can phosphorylate S6 in response to RAS/ERK pathway, serum and growth factors[168]. The latter is particularly interesting in the setting of TC that often presents mutations in genes of the MAPKinase cascade.

In our study, when we compared pmTOR, pS6 and pAKT expression in PTCs with or without *BRAF*V600E mutation, we observed no differences in pmTOR expression, higher pS6 expression, and lower pAKT expression in the *BRAF*WT when compared to *BRAF*V600E group. It seems that in PTCs harboring *BRAF*V600E mutation, the mTORC2 complex is more active in comparison to mTORC1. In a previous work of our group, it was observed a significantly overexpression of the three markers in *BRAF*V600E cPTCs compared to *BRAF*WT PTCs[210]. The

difference between these results may reflect two issues: methodological and biological. In terms of methodology, in the first study the expression of the three markers were analyzed in tissue microarrays (TMA) while in the present study we used complete histological sections. Since the immunoexpression of the markers is heterogeneous within each tumor, TMA may sometimes underrepresent the whole tumor. Another aspect to have in consideration from the biological standpoint regards the different composition of the series. In the first study the PTC series encompassed mostly cases with very good prognosis, whereas in the present study the series was enriched with PTCs carrying poor prognosis, with distant metastization and resistance to therapy. The mTOR pathway activation (and consequently the expression of its downstream effectors) may be different in these two different biological contexts.

We were also interested in evaluating if mTOR pathway was implicated on NIS expression/function in human PTCs, as it had been suggested in *in vitro* studies. We observed a significant and inverse correlation between pmTOR expression and *SLC5A5* mRNA expression, confirming for the first time in human thyroid tumors that overexpression of pmTOR may, in fact, be associated with a lower *SLC5A5* mRNA expression. Moreover, we compared pmTOR expression and the number of ^{131}I therapies and cumulative dose of RAI, and observed a significant and positive correlation between pmTOR expression and a greater number of ^{131}I therapies and cumulative dose, meaning that patients with PTCs displaying higher pmTOR expression needed more RAI therapies and were subjected to higher cumulative doses. So, pmTOR expression is associated with worse response to RAI therapy (Figure 4). These results indicate that it may be important to explore the role of mTOR in the resistance to ^{131}I therapy in order to evaluate possible advantages of pharmacological blockers of mTOR in RAI resistant PTCs.

Since we observed the mTORC2 assembly in PTCs, we explored the mTORC2 role on *SLC5A5* mRNA expression. We used a cell line derived from PTC (TPC1) and performed pharmacological blockage of mTORC1 with RAD001 and, simultaneous, mTORC1 and mTORC2 inhibition with Torin2. Both drugs were effective in terms of downregulation of

mTORC1, and Torin2 downregulated also mTORC2 downstream effectors. Albeit RAD001 caused a decrease on pS6 expression it did not alter *SLC5A5* expression, like previously observed[251]. On the contrary, Torin2 treatment caused a decrease of pS6 and pAKT expression, and was able to increase significantly *SLC5A5* mRNA expression. In TPC 1 cell line the inhibition of mTORC2 complex revealed to be of major importance in the restoration of *SLC5A5* mRNA expression. These results support the assumption that inhibition of the mTORC2 complex should be further addressed in the management of specific RAI resistant TC. Blocking of mTORC1 by rapamycin led to an increase of *SLC5A5* mRNA expression and also to RAI uptake in other TC cell lines (BCPAP and FTC133)[251] but not in TPC1[251] (and present study). All these cell lines harbor different genetic backgrounds: TPC1 harbors *RET/PTC* rearrangement, BCPAP is *BRAF*V600E mutated[210] and FTC133 is PTEN deficient[251]. Even though the authors of the study did not explore the lack of response of TPC1 cell line, we guess that, since *SLC5A5* expression is different according to the tumor genetic background[128] (and present study), such genetic differences could be the source of the aforementioned discrepancies.

*BRAF*V600E mutation is known to decrease NIS expression targeting to the membrane and this effect seems to be MAPK independent[122]. Since pAKT is overexpressed in *BRAF*V600E mutated PTCs[210], and pAKT downregulation by Torin2 caused a significant increase of *SLC5A5* mRNA expression, we may speculate that mTORC2 could be a molecular link between *BRAF*V600E mutation and NIS impairment. Further studies are needed in order to confirm or refute this hypothesis

Summing up, pmTOR is a marker of aggressiveness and a possible indicator of RAI therapy resistance in PTCs. The expression of pAKT reflects the activation of the mTORC2 complex. Nuclear translocation of pAKT may play a major role in distant metastization, and its activation seems to be involved in *SLC5A5* mRNA expression regulation (Figure 4). pAKT activation may serve as a potential marker that could help to identify the subgroup of PTCs with RAI refractory distant metastases. Moreover, the pharmacological inhibition of mTORC2 emerges as an interesting target in the management of metastatic RAI refractory PTCs.

NIS is the central molecule of TC treatment. It is postulated that downregulation/loss of function in recurrences and distant metastases causes resistance to RAI therapy, worsening considerably the patients' prognosis. In addition, it is not yet clear if NIS expression in primary tumor (mRNA and/or protein) may be useful for predicting response to RAI therapy and/or tumor behavior. There are many studies about *SLC5A5* mRNA expression and NIS protein expression in thyroid tumors, but the vast majority was performed in small series, and did not address possible associations with clinicopathological, and molecular features, nor with prognosis and response to therapy. In order to explore the aforementioned parameters, we analyzed *SLC5A5* mRNA expression and NIS protein expression in a vast series of TC (mostly DTCs) using different methodologies: quantitative real time/PCR and immunohistochemistry.

SLC5A5 mRNA expression was studied in two different series: one constituted by cases randomly selected from the archives of the Hospital de S. João and the other (used to validate our results) constituted by 378 PTCs from The Cancer Genome Atlas Database (TCGA)[99].

SLC5A5 mRNA expression was significantly lower in the tumors compared to normal adjacent tissue in both series, a finding which is in agreement with the vast majority of the studies available in the literature[222, 223, 226, 257]. This might mean that loss of expression of *SLC5A5* mRNA is a common event for all thyroid tumors, as well as an early event in thyroid carcinogenesis. Furthermore, we also observed a significantly lower *SLC5A5* mRNA expression in tumors from male gender and with vascular invasion (both are characteristics associated with clinical aggressiveness of the tumors). The analysis of TCGA data revealed that *SLC5A5* mRNA expression is significantly lower in PTCs measuring >2cm, with extensive extrathyroidal extension and in PTCs that presented a new tumor event (recurrences and/or distant metastases). The analysis of TCGA data did not confirm the association between male gender and lower *SLC5A5* mRNA expression. It was not possible to validate the association that we observed between lower *SLC5A5* mRNA expression in cases with the presence of vascular invasion because TCGA data has no information regarding vascular invasion. Nevertheless, the association observed in the analysis of TCGA data between lower *SLC5A5* mRNA expression and extensive

extrathyroidal extension directly corroborates our results. Interestingly, *SLC5A5* mRNA expression was not different between PTCS with or without lymph node metastases (a pathological feature without prognostic impact *per se*).

The association between lower *SLC5A5* mRNA expression and larger tumor size has already been described in the literature[229, 246]. Furthermore, a study in a small series of PTCs (11 PTCs) also described a lower *SLC5A5* mRNA expression in recurrent/metastatic PTCs[228] . In our series, the difference in the size was not evident since we studied mainly PTCs ≥2cm, turning impossible to perform any statistic test comparing *SLC5A5* mRNA expression between PTCs <2cm and ≥2cm. We evaluated the correlation between tumor size (as a continuous variable) and *SLC5A5* mRNA expression and did not find a significant correlation (data not shown).

To the best of our knowledge, this is the first study addressing the association of *SLC5A5* mRNA expression with a great variety of clinicopathological features and prognosis in a large series of cases. A lower *SLC5A5* mRNA expression in the primary tumor seems to be associated with higher aggressiveness and worse prognosis, being potentially useful for a risk/prognosis patient's stratification.

We also observed that the genetic background of the tumors is of major importance concerning *SLC5A5* mRNA expression. From the data obtained from the TCGA series, the presence of any of the studied mutations (*RAS, BRAF, TERT*p) caused a significantly decrease of *SLC5A5* mRNA expression in comparison to wild type PTCs (confirming our tendency regarding *BRAF*V600E mutation). The impact of *BRAF*V600E mutation in *SLC5A5* mRNA expression has been previously described, but the impact of the other mutations remained unknown. *RAS* mutated carcinomas seem to have a lower impact on *SLC5A5* mRNA expression; in fact, a previous study reported that a distinct profile of expression of genes involved in thyroid hormone biosynthesis (being *SLC5A5* one of these genes) could be observed between *BRAF*V600E and *RAS*-driven PTCs, with *RAS*-like PTCs having a relatively higher thyroid differentiation score[99]. The association between a lower *SLC5A5* mRNA expression and *TERT*p mutation was not previously

addressed; however, *TERT*p mutated PTCs needed higher number of ^{131}I therapies and were consequently exposed to higher cumulative doses[134], thus suggesting that those features may be due (among other factors) to a reduction of *SLC5A5* mRNA expression.

Regarding NIS protein expression, we confirmed the results already reported in the literature. The majority of our cases were positive for NIS expression (71.6%) and, moreover, NIS protein expression was higher in thyroid tumors compared to normal adjacent tissue, but in tumors, NIS immunostaining was mainly localized in the cytoplasm. Only 12/211 cases presented NIS in the membrane of tumor cells [224, 229, 232, 233, 235, 240, 241, 258]. Like in the study performed by Peyrottes and colleges[234], some doubts remain about the real meaning of the diffuse cytoplasmic NIS staining we have observed. These doubts were reinforced by our own findings; when we used a signal amplification system, only the membrane staining was amplified while the diffuse cytoplasmic staining has totally vanished.

The presence of NIS in the membrane of thyroid primary carcinomas was not associated with clinicopathological features, response to therapy or prognosis. Interestingly, the only aspect that those tumors had in common was that all, but one, were wild type for the studied mutations (*NRAS*, *BRAF*, *TERT*p). It is tempting to advance that the genetic background of tumors influence NIS targeting to the membrane. There are *in vitro* evidences that *BRAF*V600E mutation affects NIS targeting to the membrane [122], but the impact of the other mutations (*NRAS* and *TERT*p) had never been addressed to the best of our knowledge.

The lack of correspondence between NIS membrane staining expression and prognosis was also previously described in one study[229] . One could expect that NIS expression in primary tumor could predict NIS expression in derived recurrences and/or metastases, and consequently serve as an indicator of RAI therapy success or lack of it. This was not the case. Two reasons may explain this discrepancy: first if we look carefully to the treatment of thyroid carcinoma (surgery followed by RAI ablation), only the remnants, metastases and eventually the recurrences are subjected to RAI. Prior to RAI ablation patients are subjected to TSH stimulation, either by withdrawal of thyroid hormones or by the administration of recombinant TSH[50]. Since TSH has a major role in

NIS expression and targeting to the membrane[259], we can hypothesize that different TSH concentrations could contribute to a difference between the levels of membrane NIS in non-stimulated primary tumors and stimulated recurrences and metastases. Second, we previously observed that the genetic background is of major importance for *SLC5A5* mRNA expression and NIS protein targeting to the membrane; in a recent study from our group it was demonstrated that the genetic background of distant metastases is very often different from the one of the respective primary tumors[260]. Primary tumors, recurrences and/or distant metastases may reflect different biological conditions with different NIS expression/targeting to the membrane. This hypothesis may help to explain why NIS expression in the primary tumor does not predict RAI therapy success. In summary, NIS protein expression evaluated by immunohistochemistry presents some methodological limitations and is not informative in terms of prognosis and RAI therapy resistance being, in our opinion, of very limited value in daily practice.

Concluding remarks

In this thesis, we explored the role of the mTOR pathway in PTC and addressed possible associations with clinicopathological and molecular features, prognosis and response to therapy and found that pmTOR is a marker of aggressiveness and, possibly, of therapy resistance. The effects of pmTOR seem to be mainly mediated by mTORC2 downstream effector pAKT.

The mTORC2 complex plays a role in *SLC5A5* mRNA expression regulation: its inhibition increases *SLC5A5* mRNA expression. Furthermore, pAKT, a mTORC2 downstream effector, emerged as a possible molecular link between *BRAF*V600E mutation and *SLC5A5* mRNA expression impairment (Figure 4). Taking all this into consideration we conclude that mTOR pathway emerges as potential therapeutic target for advanced refractory DTC.

Our results demonstrated that while NIS protein expression appears to be of very limited value, *SLC5A5* mRNA expression seems to be a marker of aggressiveness and worse prognosis (Figure 4), and consequently may help in patient's prognostic stratification.

Finally, we also observed that the genetic background of the tumor is of major importance for both *SLC5A5* mRNA expression and NIS protein targeting to the cell membrane. The presence

of either *RAS* or *BRAF* and/or *TERT*p mutations caused a significantly decrease of *SLC5A5* mRNA expression. Moreover, the vast majority of DTCs with NIS expression in the membrane were wild type for the aforementioned mutations.

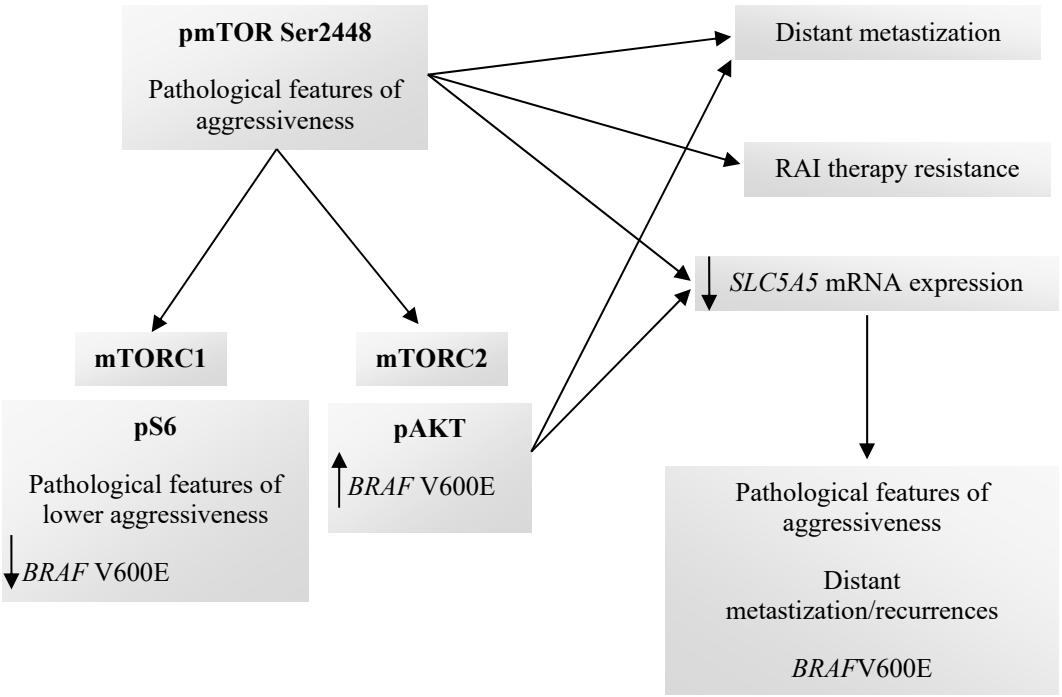


Figure 4. Schematic representation of the main conclusions of this thesis.

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Chapter 7. Appendices

7.1 – Appendix I - Paper: Genetic predictors of thyroid cancer outcome.

Paper I – Tavares C., Melo M., Cameselle-Teijeiro J.M., Soares P., Sobrinho-Simões M. (2016) ENDOCRINE TUMOURS: Genetic predictors of thyroid cancer outcome. European Journal of Endocrinology 174(4):R117-26. doi: 10.1530/EJE-15-0605

ENDOCRINE TUMOURS

Genetic predictors of thyroid cancer outcome

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Abstract

Genetic predictors of outcome are reviewed in the context of a disease – cancer – that can be (too) simplistically described as a 'successful, invasive clone of our own tissues'. Context has many faces that determine a thyroid cancer patient's outcome beyond the influence of genetic markers. There is also plenty of evidence on the prognostic meaning of the interplay between genetics and context/microenvironment factors (encapsulation, degree of invasion, staging, etc.). This review addresses only genetic alterations detected by molecular methods in surgically resected specimens, thus ruling out immunohistochemistry and (F)ISH, despite their crucial relevance as topographically oriented methods. For the sake of the discussion, well-differentiated carcinomas were divided into two main morphologic types: papillary carcinoma (classic and most variants) displaying BRAFV600E mutations and RET/papillary thyroid carcinoma rearrangements and the group of follicular patterned carcinomas that encompasses follicular carcinoma and the encapsulated form of follicular variant of papillary carcinoma, displaying RAS mutations and PAX8/PPARγ rearrangement. TERT promoter mutations have been recently described (and associated with distant metastases and reduced survival) in papillary and follicular carcinomas, as well as in poorly differentiated and undifferentiated carcinoma. TP53 mutations, previously thought to be restricted to less differentiated carcinomas, were also detected in papillary and follicular carcinoma and found to carry a guarded prognosis. Besides their putative importance for targeted therapies, the prognostic meaning of such mutations is discussed *per se* and in the setting of concurrent BRAF mutation.

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Introduction

Assuming that cancer can be defined, in an oversimplified way, as a 'highly regulated, successful invasive clone of our own tissues' or, in a less simplified but still too simplistic way, as a 'highly regulated, successful, invasive clone of our own tissues, involving a multistep accumulation of mutations in genes regulating major signalling pathways that are frequently heterogeneous genetically, epigenetically and phenotypically, as well as the cross talk of such mutations with cellular and extracellular alterations at the surrounding tissues', it does not make sense to discuss genetic predictors of thyroid cancer (or any other cancer type) outside host and surgical pathology context.

The aforementioned context has many faces that determine patients' outcomes beyond the influence of genetic markers. This applies to the age and/or gender of the patients, and the site, size and macroscopic characteristics of the cancer – namely, its pushing or infiltrative borders. The degree of invasiveness, both locally and at a distance, is measured by the TNM staging, which is the most powerful predictor of outcome of almost all cancer patients. The histological characteristics of the cancer are also a major factor of prognosis: morphological subtype, degree of differentiation, extension of necrosis, mitotic index and signs of invasion (parenchymatous, lymphovascular and to adjacent organs). The histological context can be, and frequently is, enriched by immunohistochemical data that allow to evaluate more precisely cell proliferation, overexpression (or misplacement) of oncogene products and underexpression (or, again, misplacement) of tumour-suppressor gene products and the number and the type of cells involved in the immunomodulation of cancer development.

The sort of molecular approach that immunohistochemistry provides is also achieved, and frequently reinforced, by *in situ* demonstration of gene rearrangement and gene amplification (FISH is frequently the best method to detect such genetic alterations). Both immunohistochemistry and *in situ* methods provide, furthermore, topographic information that complements the molecular data and are often crucial for understanding carcinogenesis. This has been demonstrated, for instance, by Eloy *et al*. (1) who showed that the interaction between transforming growth factor beta/Smad pathway activation and BRAF mutation plays different roles in circumscribed and infiltrative papillary thyroid carcinoma (PTC); in the latter, the interaction is associated with epithelial-to-mesenchymal transition and local invasion, as well as to nodal metastization of infiltrative PTCs (1).

Thyroid carcinomas are classified according to the cell type they derive from, their degree of differentiation and their cytoarchitecture. Follicular cell-derived tumours comprise well-differentiated thyroid carcinoma (WDTC), poorly differentiated thyroid carcinoma (PDTC) and undifferentiated thyroid carcinoma (UTC). The well-differentiated group encompasses, according to cytoarchitecture and nuclear features of the neoplastic cells, follicular thyroid carcinoma (FTC) and PTC, with the latter having two main variants: classic PTC (cPTC) and follicular variant PTC (FVPTC). The minority of carcinomas that derive from parafollicular C cells are named medullary thyroid carcinoma (2).

In this review, we will just focus on genetic alterations detected by molecular methods in surgically resected specimens, thus skipping their usefulness in cytopathology. To keep the paper within an adequate size, we will only address the importance of the genetic predictors of outcome of patients with follicular cell-derived carcinomas displaying good or moderate differentiation, thus avoiding medullary carcinoma and UTC. PDTC will be discussed together with the respective better differentiated counterparts PTC and its variants, namely, FVPTC and FTC.

Clinico-pathological factors vs genetic predictors of outcome

In a recent article on the usefulness of molecular biomarkers in thyroid cancer, we concluded that, for the moment, clinical and histopathological prognostic factors remain much more important than genetic factors for diagnostic and prognostic purposes (3). This conclusion is, however, challenged almost every day by the publication of new molecular data in the different types of thyroid cancer. The most important of such publications was the 'Integrated genomic characterization of papillary thyroid carcinoma' that provided a detailed description of the genomic landscape of 496 cases of PTC under the auspices of The Cancer Genome Atlas (TCGA) Research Network Initiative (4).

Besides a huge amount of genetic and epigenetic information that will take time to fully understand, it is interesting to realize that the aforementioned study (4) confirmed the existence of two main genetic types of differentiated thyroid carcinoma (DTC) that correspond to cPTC (and some variants of PTC such as the tall cell and Warthin-like variant) and to the group of follicular

patterned carcinomas that encompass FVPTC, as our group and others have suggested years ago (5, 6, 7). The absence of solid prospective studies on thyroid cancer and the close relationship between clinical, pathological, immunohistochemical and genetic factors turn very difficult to discuss out of the global context the prognostic role played by the latter (8).

Of the numerous genetic alterations detected in WDTC and PDTC, we included in the present review those that are more prevalent and/or seem to play a more important prognostic role. It is the case of BRAF, RAS, TERT promoter and TP53 mutations and of RET/PTC and PAX8/PPARγ rearrangements.

RET/PTC and *PAX8/PPARγ* rearrangements

RET/PTC rearrangements are quite frequent in PTC, whereas PAX8/PPARγ rearrangement is often detected in follicular patterned lesions (FVPTC and FTC) (3, 5, 6, 7, 9); the overall evidence indicates that tumours with either of these rearrangements rarely evolve to less differentiated forms (i.e. their prevalence is very low in PDTC and UTC). *RET/PTC* is a chimeric gene generated by the fusion of the RET tyrosine kinase (TK) domain with the 5′ terminal region of genes that are constitutively expressed in thyroid follicular cells (10) allowing dimerization of the RET TK domain and its constitutive activation. The most frequent forms of this oncogene in PTC are *RET/PTC1* and *RET/PTC3*, both arising from chromosome 10 inversions (11). *RET/PTC1* rearrangement appears to be associated with small, classic type PTC displaying low proliferation and occurring in young patients (12, 13, 14, 15). At variance with this, *RET/PTC3* rearrangement is prevalent in the solid variant of PTC that is frequent in children and was often found in PTCs occurring in the setting of the Chernobyl accident (16), being more prone to a more aggressive behaviour (13, 14, 15, 17). Despite being associated with signs of clinical aggressiveness (namely nodal and lung metastases), cases of solid variant of PTC arising in young patients, with or without *RET/PTC3* rearrangement, respond well to radioactive iodine (RAI) treatment and are not significantly associated with a worse survival of the patients.

Taking the data on record in the literature as well as our own experience into account, it may be concluded that the prognostic value of *RET/PTC* rearrangement in thyroid cancer has not been fully clarified yet.

*PAX8/PPARγ*rearrangement has been associated with some adverse prognostic features (e.g. multifocality and vascular invasion) in some series, but the gathered evidence is not strong enough to identify this rearrangement as a genetic predictor of outcome in thyroid cancer (9, 18). Furthermore, *PAX8/PPARγ*rearrangements have been also detected in 14% of the cases of follicular thyroid adenoma (FTA) (19).

RAS mutations and prognosis

RAS are small GTPase-proteins that act as a molecular switch propagating signals from TK and non-TK receptors and activating the MAPK and other signalling pathways. RAS mutations are more prevalent and seem to be more relevant as a prognostic indicator in follicular patterned lesions (FVPTC, FTC and, namely, PDTC) than in cPTC (18). All of the three RAS genes (H, K and N-RAS) were shown to be mutated in both benign and malignant thyroid tumours but the frequency of the mutations is higher in FTC (36%), PDTC (55%) and UTC (52%) and more frequently affects the N-RAS gene (20).

RAS mutations are less prevalent in benign and malignant Hürthle cell tumours (5 and 11% respectively) than in their non-Hürthle cell counterparts and less prevalent in PTC (10%) than in FTC (25–30%) (7, 20). Within PTC, RAS mutations are rare in its classic form, whereas in FVPTC, its prevalence falls within the range of other follicular patterned tumours (~25%) (6).

The controversy on the prognostic value of RAS mutations in thyroid cancer results partially, at least, from the fact that RAS mutations are present along all of the whole spectrum of thyroid lesions, from FTA to the deadly UTC. Garcia Rostan *et al*. (21) have shown that patients with RAS mutated carcinomas, namely PDTC, harbour distant metastases more frequently and have higher mortality, being RAS mutations an independent predictor of poor survival (21). Other studies disclosed a similar association between (N) RAS mutation and distant metastases and/or lower survival in FTC (22, 23).

The assumption that RAS mutations can predispose to differentiation loss in thyroid cancer derives from their presence in DTC with areas of dedifferentiation and from their greater prevalence in PDTC and UTC than in DTC (24).

It has been difficult to demonstrate the prognostic value of RAS mutations due to the relatively small size of the majority of the series (in particular concerning FTC, PDTC and UTC that are less frequent than PTC) and the too short follow-up in most situations. Large, multicentric studies will be necessary to establish definitely the prognostic value of RAS mutations.

BRAF and NIS expression

BRAF gene encodes a serine/threonine kinase that belongs to the RAS–RAF–MEK–ERK–MAP kinase pathway, whose biological role is to mediate cellular responses to growth factors. There are several BRAF mutations, the BRAFT1796A (in exon 15) is largely the more prevalent, leading to a substitution of a valine by a glutamic acid at position 600. Such a mutation causes increased BRAF kinase activity and the subsequent phosphorylation of MEK1/2 and ERK1/2, turning the activation of the MAP kinase pathway independent from upstream factors activation (25).

BRAFV600E mutation is the most prevalent point mutation in PTC, being present in 36–83% of cases. It rarely co-exists with other prevalent genetic events such as RET/PTC rearrangement or RAS mutation (18). BRAFV600E mutation exhibits a strong genotype–phenotype association; it is (almost) exclusively detected in PTC exhibiting a papillary or mixed follicular/papillary growth pattern, regardless of being a cPTC or any of the PTC variants (other than the encapsulated FVPTC) (5).

Besides the frequent BRAFV600E mutation, other alterations were detected in the BRAF gene in PTCs: the BRAFK601E mutation, which occurs mainly in FVPTC (<10% of the cases) (5), and the in-frame deletion VK600-1E that has been detected in rare cases of solid variant of PTC. BRAF rearrangements, namely the AKAP9–BRAF fusion, were also described as rare events preferentially found in radiation-induced PTC (18). At present, there is not enough evidence to evaluate the putative prognostic role of the aforementioned rare BRAF alterations.

Although functional studies, using thyroid-targeted BRAFV600E transgenic mice (26) and BRAFV600E transfected thyroid cell lines (27), indicate that BRAF mutations lead to a more 'aggressive type' of PTC, several other studies, addressing the correlation between BRAFV600E and the clinical features of PTC, provided discrepant results (see below).

Some studies reported significant associations between BRAF mutation and poor prognostic indicators like older age (28, 29), male gender (30, 31), extrathyroid extension (28, 32), regional metastases (29, 32), distant metastases (33), higher tumour staging (28, 32, 33), tumour size (31, 34, 35) and tumour recurrence (32, 36). Other studies have not observed the aforementioned associations (37, 38, 39). Furthermore, Elisei *et al.* (40) have demonstrated that the search for BRAFV600E mutation may prove useful to modulate the treatment among low-risk PTC patients, those who require less or more aggressive treatment. Recently, a multicenter retrospective study showed that BRAFV600E was significantly associated with increased cancer-related mortality among patients with PTC, but the association was not independent of several clinico-pathological features of aggressiveness (41).

We observed that BRAFV600E PTCs tended to occur in older patients and did not exhibit a significant association with signs of clinico-pathological aggressiveness – namely larger size, extrathyroidal extension, vascular invasion and lymph node metastases (5, 8) – or poor circumscription (8). This does not mean, however, that BRAF mutation cannot contribute for progression of PTC toward less differentiated carcinomas in the appropriate context, because our group and others (28, 33, 42) detected BRAFV600E mutation in 10–35% of UTC.

Despite the BRAF mutation controversial association with guarded prognostic features, its association with a decrease in expression of several 'thyroid specific genes' or 'iodine handling genes' (36, 43, 44) is widely acknowledged. The association of BRAF mutation with the loss of RAI avidity in recurrent PTC has been confirmed *in vitro* and *in vivo* (36, 45). It was recently shown that MEK inhibition may restore RAI incorporation, turning BRAF and/or MEK inhibitors into promising targets to treat RAI-refractory thyroid cancers (45, 46).

TERT promoter mutations

About two-thirds of thyroid carcinomas display telomerase activation that is more frequent in UTC than in DTC (42). Capezzone *et al.* (47) observed telomerase activity in most sporadic and familial malignant thyroid tumours, as well as in some adenomas. Recently, mutations in the promoter region of the telomerase (*TERT*) gene were reported in follicular cell-derived thyroid carcinomas (FCDTC) (48, 49, 50). These mutations occur in two hotspot positions, located at −124 and −146 bp upstream from the ATG start site (−124G>A and −146G>A, C>T on opposite strand) and confer enhanced *TERT* promoter activity, putatively by generating a consensus-binding site (GGAA) for ETS transcription factors within the *TERT* promoter region (51).

In a large series of 469 carcinomas, we found *TERT* promoter mutations in 7.5% of PTC, 17.1% of FTC, 29.0% of PDTC and 33.0% of UTC (52). This stepwise increase in the frequency of *TERT* promoter mutations from well to poorly differentiated and undifferentiated carcinomas was also reported in other studies (49, 50). No *TERT* promoter mutations were found in normal tissues, benign lesions or

medullary thyroid carcinomas. Like RAS mutations, the frequency of *TERT* promoter mutations seems to be lower in tumours with oncocytic features than in their non-oncocytic counterparts; these observations reinforce the assumption that oncocytic tumours have a different set of molecular alterations and probably also alternative mechanisms for cell survival (53, 54, 55). The majority (about 80%) of mutated cases present the −124G>A mutation. In PTC, *TERT* promoter mutations were significantly more frequent in *BRAF* mutated tumours (50, 52). *TERT* promoter mutations were associated with increased mRNA expression, and this increase was more pronounced in tumours harbouring both *BRAF* and *TERT* promoter mutations (48).

Several studies analysed the relationship between *TERT* promoter mutations and clinico-pathological features (49, 50, 52, 56, 57), and four studies also analysed the implications of the presence of these mutations on patients' clinical outcomes (52, 56, 58, 59). *TERT* promoter mutations were associated with older age of the patients at diagnosis, larger tumour size, distant metastases and a higher stage in several studies (50, 52, 57). The association with distant metastases seems to be particularly consistent and has been reported in most of the studies, strongly suggesting that there is a link between *TERT* promoter mutations and the metastatic potential of FCDTC. From the clinical standpoint, this association is extremely relevant because distant metastases are major determinants of prognosis, especially in older patients (60).

In our study (52), patients with DTC harbouring *TERT* promoter mutations were less prone to be disease free at the end of follow-up, and similar results were found in three other studies (56, 58, 59). Our study also showed that patients with *TERT*-mutated tumours were submitted to more treatments with radioiodine with higher cumulative doses, as well as to other treatment modalities like surgery for recurrent disease, external beam irradiation or treatment with TK inhibitors (52). Furthermore, patients with tumours harbouring *TERT* promoter mutations had increased disease-specific mortality, and this finding was independent of age and gender (52).

As previously mentioned, *TERT*-mutated PTC harbours more frequently *BRAF* mutations than *TERTwt* tumours. Horn *et al.* (51) advanced that the mutation creates newly consensus binding sites for *TCF* subfamily transcription factors (Elk1 and Elk4) that can be activated by *BRAF*. Our results in *TERT* mRNA expression corroborated this assumption, showing an increased *TERT* expression in tumours harbouring *BRAF* and *TERT* mutation (48). Because BRAF has also been associated with worse prognosis in some studies, several authors hypothesized that both mutations could cooperate toward a worse prognosis (50, 61). One still ignores the mechanism behind the putative cooperation between BRAF and TERT promoter mutation. It is nevertheless tempting to speculate, considering the pro-senescent effect of BRAF mutation alone (62), that TERT promoter mutations may contribute to abrogate such effect through their, role leading to evasion from senescence (63, 64, 65). Taking into account that the prognostic value of BRAF is currently under debate and that *TERT* promoter mutations were independently associated with aggressive clinico-pathological features and worse outcome in all of the large series published to date (66), we think that, at present, the most important question is to clarify, whether or not, after controlling the clinical importance of *TERT* mutations, *BRAF* goes on adding a significant prognostic value (66). Multicentric studies with large series of patients will be necessary to clarify if the 'addition' of BRAF mutational status to a TERT-mutated tumour has indeed value for prognostic stratification (66).

TP53 mutations

Most *TP53* mutations lead to the expression of a mutant protein or, less commonly, to its absence (67, 68). In thyroid carcinomas, *TP53* mutations are not different from those of cancers at other sites and have been described in exons 5–9, with 273 being the codon most often altered (42, 67, 69, 70, 71, 72, 73). No p53 expression or mutation has been found in normal thyroid or in benign lesions, including follicular adenoma, adenomatous goitre and chronic thyroiditis (72, 73, 74, 75, 76). For years it was repeated that more than 98% of DTC (PTC and FTC) had a normal *TP53* gene (18, 69, 70, 71, 72, 73, 75, 77), even when cases secondary to radiation exposure were included (78). This scenario may be changing due to the utilization of next-generation sequencing; using this methodology, Nikiforova *et al.* (79) reported the presence of TP53 mutations in 3.5% of PTC (2/57) and in four of 36 FTC (11.1%); the four FTC cases were oncocytic carcinomas and three were widely invasive (75). In the recent TCGA study (4), TP53 mutations were detected in 0.7% of PTC thus confirming their scarcity in PTC, but no clinico-histopathological data were provided on the mutated cases. The results of the study by Nikiforova *et al.* (75) study regarding the high clinical aggressiveness of TP53 mutated DTC fit with previously reported results. A small proportion of aggressive PTC are associated with *TP53* mutations and/or p53 expression; the tall cell variant of

PTC is associated with a significantly higher rate of p53 than common PTC (80). Positivity for p53 protein has been detected in rare aggressive thyroid tumours such as a mixed columnar and tall cell variant of PTC (81) and a squamous cell carcinoma associated with the tall cell variant of PTC (82). Positivity for p53 protein has also been reported in some aggressive cases of the cribriform–morular variant of PTC (83, 84). Immunohistochemical evaluation of the columnar cell variant of PTC showed a predominantly weak nuclear p53 staining in both indolent and aggressive tumours (81).

Loss of cellular polarity/cohesiveness, hobnail features and micropapillary structures, either alone or in combination, are independent predictive factors for lymph node metastasis both in common PTC and in papillary microcarcinoma (82, 83, 84, 85). All of these peculiar morphological features are characteristic of the so-called micropapillary/hobnail variant of PTC (85, 86, 87, 88, 89), an aggressive type of PTC carrying poor outcome, which is consistently positive for p53 (85, 86, 87, 89) at the immunohistochemical level. Hobnail features were most commonly observed in association with PDTC and UTC (90). These features have also been associated with other histologic variants that are known to be more clinically aggressive, such as increased mitotic activity and/or necrosis and lymph node metastases at presentation. It has therefore been suggested that hobnail features may be a manifestation of 'higher-grade transformation' (90). The recent observation by our group (91) of two fatal cases of the micropapillary/hobnail variant of PTC positive for p53 by immunohistochemistry (Fig. 1) and TP53 mutated at the molecular level with progression to UTC supports the involvement of p53 in such transformation (90, 92). Inactivating TP53 mutations have been reported in about 26% of PDTC (71, 73, 85) and in more than 60% of UTC (42, 69, 70, 71, 73, 85). The results of the studies based on the detection of nuclear accumulation of p53 protein (73, 74, 75, 76, 86, 87) fit with molecular studies. p53 expression is more obvious in areas showing active infiltrative growth and/or at the periphery in PDTC, and widespread positivity for p53 is characteristic of UTC. The analysis of *TP53* mutations and/or p53 expression in PTC co-existing with UTC has shown that p53 expression/mutation is limited to the undifferentiated components (70, 71, 86, 88). Moreover, re-expression of WT p53 in human UTC cell lines with a mutated p53 has been associated with re-expression of the paired box domain transcription factor Pax-8, thyroglobulin, thyroperoxidase and TSH receptor (72, 89). All of these findings strongly support that *TP53* inactivation plays an important role in the progression from differentiated to undifferentiated carcinoma, as a final event in the tumourigenic process, contributing to the highly aggressive phenotype of these tumours (90).

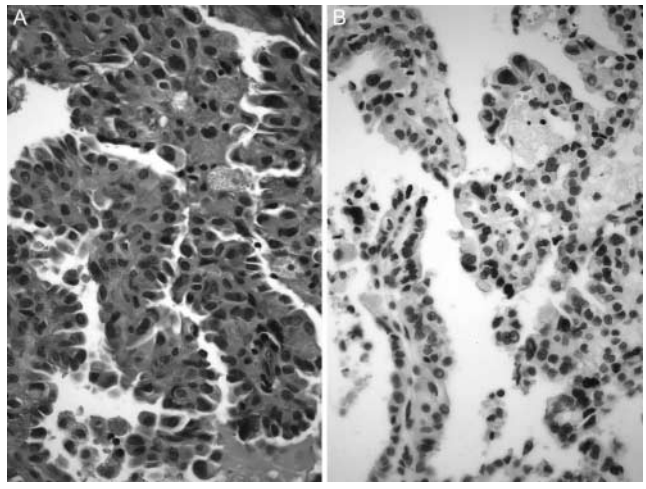

Figure 1
Micropapillary/hobnail variant of PTC. (A) The papillary structures are lined by cells with dense eosinophilic cytoplasm and the nuclei placed in the apex of the cytoplasm producing a surface bulge (hobnail appearance). There are also areas of cellular discohesiveness and micropapillary pattern (H&E, 400×). (B) The nuclei of the tumour cells show strong positivity for p53 (clone DO-7, Dako, Denmark, 400×).

miRNA and lncRNA in thyroid cancer outcome

Of the numerous molecules and mechanisms described in recent years in the oncology field, miRNA and lncRNA arise as major players due to their action on the modulation of known cancer genes and/or their products (oncogenes, tumour suppressor genes and apoptotic proteins).

It has been hypothesized that some of the miRNA and/or lncRNA (or a set of) can help in the differential diagnosis of benign and malignant tumours, however scarce information is available regarding their putative role on prognosis. Nevertheless, some miRNA have been repeatedly found dysregulated in thyroid cancer, in particular in PTC (miR-146b, miR-181b, miR-187, miR-221 and miR-222) and the same set of molecules has been associated with tumour aggressiveness in some studies (92). Unfortunately, the relevant set of miRNAs varies from one report to the other, turning difficult or even impossible to draw, at present, any meaningful conclusions.

The same holds true concerning the available data on lncRNAs. The complexity of the available evidence is huge because these long (longer than 200 nt) RNAs can play a role at both the transcriptional and the post-transcriptional gene regulation level. lncRNAs NAMA, AK023948 and PTCSC3AA (PTC susceptibility candidate 3) are among the (yet) reduced number of lncRNAs that have been associated with PTC (93, 94). Until now it has not been possible to ascertain any role to lncRNA in the prognosis of thyroid cancer patients.

Final remarks

The importance of genetic markers for predicting thyroid cancer outcome is limited by the pre-eminence of clinical, histopathological, immunological and other context-driven features. Despite this, there is enough evidence to claim that TERT promoter mutations and TP53 mutations are major molecular biomarkers of prognosis and to suggest that BRAF and RAS mutations may also play a prognostic role in some conditions. Besides prognosis, the aforementioned mutations and the respective molecular pathways, as well as other genetic and epigenetic alterations recently identified by the Cancer Genome Atlas (4), will probably serve as targets for the so-called personalized therapy.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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7.2 – Appendix II. UICC/AJCC staging system for differentiated thyroid carcinoma (8th edition).

Table 1 UICC/AJCC staging system for differentiated thyroid carcinoma

Adapted from UICC/AJCC TNM 8th edition 2017[91]

T- Primary Tumor

T1- Tumor ≤2cm in greatest dimension limited to the thyroid

T1a- Tumor <1cm, limited to the thyroid

T1b-Tumor >1cm but ≤2cm in greatest dimension limited to the thyroid

T2- Tumor >2cm but ≤4cm in greatest dimension limited to the thyroid

T3a-Tumour > 4 cm in greatest dimension, limited to the thyroid

T3b-Tumor of any size with gross extrathyroidal extension invading only strap muscles (sternohyoid, or omohyoid muscles)

T4a*-Tumor of any size extending beyond the thyroid capsule to invade subcutaneous soft tissues, larynx, trachea, esophagus or recurrent laryngeal nerve

T4b*-Tumor invade prevertebral fascia or encases carotid artery or mediastinal vessels

N- Regional lymph nodes

Nx- Regional lymph nodes cannot be assessed

N0- No regional lymph node metastases

N1a- Metastases to Level VI (pretracheal, paratracheal, and prelaryngeal/Delphian lymph nodes

N1b- Metastases to unilateral, bilateral, or contralateral cervical (Levels I, II, III, IV, or V) or retropharyngeal or superior mediastinal lymph nodes (Level VII)

M- Distant metastases

Mx- Distant metastases cannot be assessed

M0- No distant metastases

M1- Presence of distant metastases

Staging

Stage<55 year sold		Stage ≥55 years old	
Stage I		Stage I	
	Any T. Any N. M0		T1a, T1b. N0. M0
Stage II		Stage II	
	Any T. Any N. M1		T3. N0. M0
			T1, T2, T3. N1. M0
		Stage III	
			T4a. Any N. M0
		Stage IVa	
			T4b. Any N. M0
		Stage IVb	
			Any T. Any N. M1

*All anaplastic thyroid carcinoma are considered as T4