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Evaluation of *RRAS2* and *ACD* Promoter Mutations in Thyroid Tumours

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Evaluation of *RRAS2* and *ACD* Promoter Mutations in Thyroid Tumours

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Resumo

O cancro da tiroide é a neoplasia endócrina mais frequente, sendo a décima mais prevalente em ambos os sexos e apresentando um bom prognóstico global.

A Ras related 2 (*RRAS2*), também conhecida como TC21, é uma proteína de ligação ao GTP que, juntamente com a *RRAS1* e a *RRAS3*, faz parte da subfamília das *RRAS* GTPase. As mutações no gene *RRAS2* no hotspot de cauda longa Q72L/H são responsáveis pelo bloqueio da hidrólise do trifosfato de guanosina (GTP) nas proteínas da superfamília Ras, gerando proteínas constitutivamente ativas que se ligam preferencialmente ao GTP. Em modelos de murganho, a indução da modificação Q72L/H é responsável pela ocorrência de tumores da tiroide. Este gene é composto por cinco exões que codificam um membro da superfamília Ras que participa na via RAS-MAPK.

O gene *ACD* codifica a proteína de ligação ao telómero TPP1 que recruta a telomerase para os telómeros. Quando falamos do promotor deste gene, este é normalmente designado por promotor TPP1 (*TPP1p)*. A proteína TPP1 desempenha um papel fundamental na regulação da estabilidade e no comprimento dos telómeros. Foi descrito que as mutações no promotor do gene *ACD (TPP1p)* criam novos locais de ligação de fatores de transcrição, tal como apresentado anteriormente para o promotor da telomerase (*TERTp*). A co-expressão destes dois genes leva ao alongamento dos telómeros, indicando que mutações no *TPP1p* e *TERTp* podem cooperar para a imortalização de células cancerígenas.

O nosso projeto teve como objetivo avaliar mutações no hotspot Q72L do gene *RRAS2* e no *TPP1p* em tumores da tiroide. Após a genotipagem do *RRas2*, concluímos que a presença de mutações no hotspot Q72L/H pode não representar um evento oncogénico frequente em tumores da tiroide contrariamente ao reportado por outros. Para a *TPP1p*, embora já apresentada na literatura, estas alterações não foram detetadas na nossa série, apontando para que possam também ser um evento raro nos tumores da tiroide.

Palavras-chave: Cancro da tiroide; gene *RRAS2*; hotspot Q72L/H; promotor da telomerase (*TERTp*); promotor TPP1 (*TPP1p*).

Abstract

Thyroid cancer is the most frequent endocrine neoplasm, being the tenth most prevalent in both genders and presents an overall good prognosis.

Ras related 2 (*RRas2*), also known as TC21, is a GTP-binding protein that together with *RRas1* and *RRas3*, is part of the RRas GTPase subfamily. Mutations in *RRAS2* gene in the long-tailed hotspot Q72L/H block the hydrolysis of guanosine triphosphate (GTP) in Ras superfamily proteins, generating constitutively active proteins that will preferentially bound to GTP. In mice models, the insertion of the mutation *RRas*2 Q72L/H is associated to the occurrence of thyroid tumours. This gene is composed by five exons encoding a member of the Ras superfamily that participates in the RAS-MAPK pathway.

The *ACD* gene, also known as *TPP1*, encodes the telomere-binding protein TPP1 which recruits telomerase to telomeres. When addressing the promoter of this gene, we refer to it as the TPP1 promoter (*TPP1p*). TPP1 protein plays a key role in the telomere stability and length regulation. Mutations in its promoter were reported to create novel transcription factor binding sites as previously presented for telomerase promoter (*TERTp*). Co-expression of these two genes lead to telomere elongation, indicating that mutations in the *TPP1p* and *TERTp* can cooperate for the immortalisation of cancer cells.

Our project aimed to evaluate mutations in the Q72L hotspot of the *RRAS2* gene and in the *TPP1p* in thyroid tumours. Following genotyping of *RRas2*, we conclude that the presence of mutations in the Q72L hotspot may not represent a frequent oncogenic event, as we did not detect them. For TPP1p, although already presented in the literature, these alterations were not detected in our series, suggesting that they may be a rare event in thyroid tumours.

Keywords: Thyroid cancer; *RRas2* gene; Q72L/H hotspot; Telomerase promoter (*TERTp*); TPP1 promotor (*TPP1p*).

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List of abbreviations

ACD	Adrenocortical dysplasia protein homolog
Akt	Protein kinase B
ALK	ALK Receptor Tyrosine Kinase
AP	Anteroposterior diameter
ATC	Anaplastic thyroid carcinoma
ATR	Ataxia telangiectasia and Rad3
BRAF	B-Raf Proto-Oncogene, Serine/Threonine Kinase
CHUSJ	Centro Hospitalar e Universitário de São João
DC	Dyskeratosis congenita
ddNTPs	Dideoxynucleotides
DM	Diabetes Mellitus
dNTPs	Deoxyribonucleotide
DSB	Double-strand breaks
DTC	Differentiated thyroid carcinoma
ECA	External carotid artery
EKR	Extracellular signal-regulated kinase
FTC	Follicular thyroid carcinoma
G	Glutamine
GDP	Guanosine diphosphate
GTEX	Genome-Tissue Expression
GTP	Guanosine triphosphate
H	Histidine
IAT	Inferior thyroid arteries
IMA	Inferior mesenteric artery
KRAS	KRAS Proto-Oncogene
L	Leucine
MAPK	Mitogen-Activated Protein Kinase
MEN2	Multiple endocrine neoplasia type 2
MTC	Medullary thyroid carcinoma
NRAS	NRAS Proto-Oncogene
NS	Noonan syndrome
nTPM	Normalised transcripts per million
OB	Oligonucleotide/oligosaccharide binding fold domain
OC	Oncocytic carcinoma
OS	Overall survival
PAX8	Paired box 8
PCR	Polymerase Chain Reaction
PDTC	Poorly differentiated thyroid carcinoma
PKC	Protein kinase C
POT1	Telomere protection 1
PTC	Papillary thyroid carcinoma
PTEN	Phosphatase And Tensin Homolog
RAP1	Repression/activation protein 1
RET	RET protooncogene
RRAS	Ras related subfamily
RRAS2	Ras related protein 2
rT3	Reverse T3
RTKs	Receptor tyrosine kinase
SNV	Single nucleotide variant
STA	Superior thyroid arteries
T3	Triiodothyronine
T4	Thyroxine
TBD	C-terminal TIN2-binding domain
TC	Thyroid cancer
TCGA	Cancer Genome Atlas
TERTp	Telomerase Reverse Transcriptase promoter
Tg	Thyroglobulin
THPA	The Human Protein Atlas
THs	Thyroid hormones
TIN2	Nuclear factor interacting with TRF1 2
TP53	Tumour Protein P53
TPM	Transcripts per million
TPP1	TIN2-POT1 organising protein
TPP1p	TPP1 promoter
TRF1	Telomeric repeat binding factor 1
TRF2	Telomeric repeat binding factor 2
TRH	Thyrotropin-releasing hormone
TSH	Thyroid stimulating hormone
WDTC	Well-differentiated thyroid carcinomas
WHO	World Health Organization

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1. Introdution

1.1. Thyroid Gland

The thyroid gland is an endocrine organ located in the lower part of the neck, containing two symmetrical lateral lobes, the right one being slightly larger than the left, connected by a structure called the isthmus, which passes in front of the trachea and is located in the midline of the neck (Figure 1). Each lobe has an upper and lower part, but sometimes there may be a third lobe, the pyramidal lobe, which appears before the isthmus (1, 2). The size of the gland varies according to several factors: age, sex, physiological state, race and geographical location; curiously, it is comparatively larger in females than in males (1). The larger size of the gland in women is due to the fact that there is greater production and release of hormones when they are in their period of mestruation and previous studies have stated that during the periods of menarche and menopause, the size of the thyroid is larger than in men (3). The dimensions of the lobes are not equal along individuals life, normally in newborns they can have a size of 8–9 mm anteroposterior diameter (AP), in infants aged one year 12–15 mm AP and in the adult population between 13–18 mm AP. With regard to the volume of a normal thyroid, males have a higher value, 12–19 ml, than females, 10–15 ml (4). It is the first endocrine organ to form during foetal development, starting at four weeks gestation as an epithelial diverticulum arising from the endoderm of the foregut near the base of the primitive tongue and reaching its final shape by the seventh week of gestation (5). This gland has a rich vasculature through the external carotid artery and the subclavian artery, which in turn give rise to the superior thyroid arteries (STA) and the inferior thyroid arteries (IAT). Venous drainage is via the superior and middle thyroid veins which drain into the internal jugular vein, and the inferior thyroid veins which drain into the brachiocephalic vein. Knowing that STA initially originated from the external carotid artery, studies show that STA must also originate not only from the external carotid artery (ECA) but also from the common carotid artery and its bifurcation (6). In addition, there is the inferior mesenteric artery (IMA), arising from the aortic arch or innominate artery, which is found in approximately 3% of patients and may be enlarged in patients presenting with goitre (2).

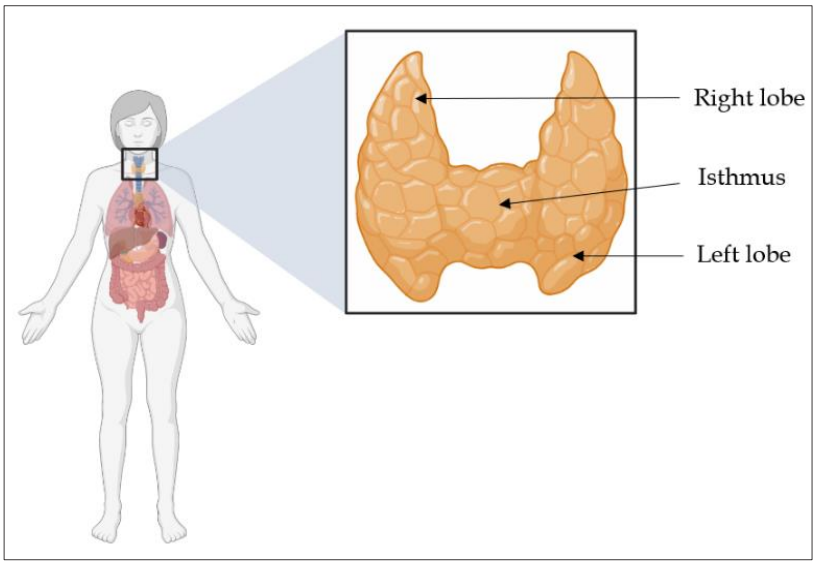


Figure 1. Illustration of the location, shape and identification of thyroid structures. Source: BioRender.com

1.2. Thyroid Function and Hormonal Regulation

The structural and functional unit of the thyroid are the thyroid follicles that are composed of follicular cells, presenting a cubic or columnar structure and surrounded by follicles lined by these cells containing inside the colloid, a gelationous and viscous fluid (7). In addition to the follicular cells, this gland contains parafollicular cells, also known as C cells (Figure 2), located between the thyroid follicles and have the function of synthesising and secreting calcitonin, responsible for establishing normal calcium levels in the body (8). Thyroid follicular cells are responsible for the production and release of thyroid hormones, triiodothyronine and thyroxine, T_3 and T_4 respectively, playing a key role in regulating individual's metabolism. Thyroid hormones (THs) are endocrine messengers that are involved in cell differentiation, development, and metabolism as well as in energy stability and homeostasis. Biologically, THs have roles in shaping gene expression in highly regulated events. THs signalling involves active transport of TH into the target cell, binding of triiodothyronine (T_3) to the intracellular thyroid hormone receptor (TR), to regulate target gene transcription (9). Thyroid hormone release is tightly regulate by a negative feed back mechanism that involves the hypothalamus- pituitary-thyroid axis. The hypothalamus must be stimulated to release thyrotropin-releasing hormone (TRH). This stimulates the pituitary gland to produce thyroid stimulating hormone (TSH), that enters the bloodstream and binds to receptors in the follicular cells. After TSH binds to the membranar receptors, follicular cells are stimulated to several processes including the take up iodine from the blood. In this way, the iodine that has been captured is oxidised by the enzyme thyroperoxidase and incorporated into thyroglobulin (Tg), a protein that is produced by the follicular cells. Thyroglobulin is stored in the central part of the follicle as a macromolecule (colloid). When the body needs thyroid hormones, Tg is internalised by the follicular cells and the hormones T_4 and T_3 are

released from the thyroglobulin residues and thus processed and secreted into the bloodstream. As the levels of T_3 and T_4 in the blood increase, there will be inhibition of TRH secretion by the hypothalamus and TSH secretion by the pituitary gland, thus reducing thyroid stimulation. This negative feedback mechanism will help regulate thyroid hormone production according to the body's needs (12). It is well-described from serum evaluation of these hormones that there is an association between thyroid dysfunction and adverse health problems, such as, mental retardation, obesity, metabolic disorders, cardiovascular disease, and sexual function (13). The refered hormonal dysregulation or dysfunction of thyroid hormones are frequently related with specific diseases characterized by hypothyroidism or hyperthyroidism that we will further discuss (15).

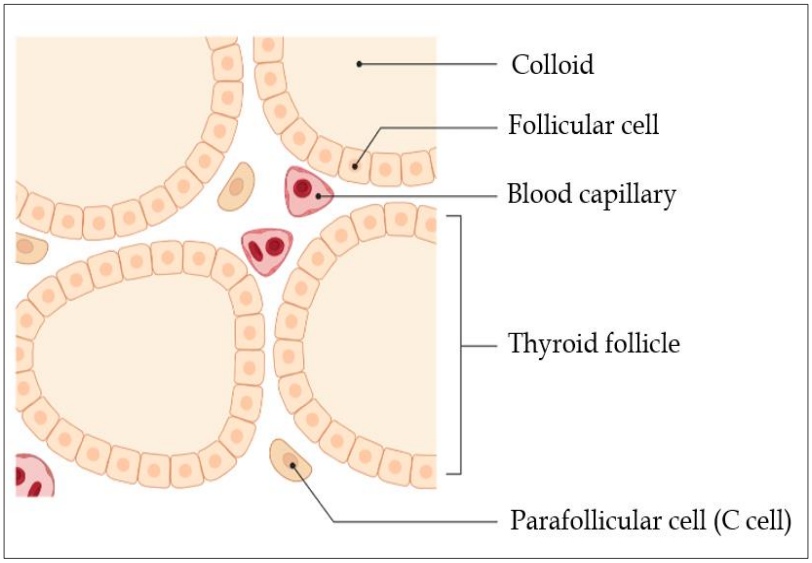


Figure 2. Illustration of thyroid functional structure. Source: BioRender.com

1.3. Phisiopatholagy

Thyroid disorders are one of the most prevalent endocrine disorders in the world, second only to Diabetes Mellitus (DM) in endocrine organs. It is estimated that there are 750 million people worldwide who have thyroid disorders, including thyroid nodules, hyperthyroidism and hypothyroidism (9). Hyperthyroidism is characterized by the overproduction of T3 and T4 and a decrease in TSH, causing heat intolerance, weight loss, anxiety and diarrhoea. It has some less specific symptoms, such as increased basal metabolic rate, thermogenesis and cardiac output (10, 11). On the other hand, hypothyroidism is characterised by low levels of the hormones triiodothyronine and thyroxine and is associated with certain symptoms such as fatigue, weight gain, an increased sensitivity to cold temperatures and muscle weakness. Both hyperthyroidism and hypothyroidism arise frequently in the setting of autoimmune thyroid diseases, being Graves disease the most frequent cause of hyperthyroidism and Hashimoto thyroiditis the most commom cause of hypothyroidism (12). However, only approximately 5% of thyroid

nodules are malignant, the remainder being colloid nodules, cysts and thyroiditis (80%) and benign follicular neoplasms (10–15%) (13).

1.4. Thyroid Nodules

Thyroid nodules are common in the general population and are usually benign. Thyroid nodules are in the majority of the cases an increased growth of cells, leading to the formation of a lump in the gland. Epidemiologically, 3% to 7% of the world's population has a palpable nodule, with a prevalence of 70% when patients are screened by ultrasound but less than 5% are malignant. Nodules larger than one centimetre are subject to evaluation, only if there is any suspicious for malignancy or an identified risk factor. Some of the factors for nodule screening for risk assessement include: family history of cancer or thyroid disease, pior radiation treatment to the head and neck (14), or familal syndromes associated with thyroid cancer such as multiple endocrine neoplasia type 2 (MEN2) (i.e., RET protooncogene (RET) germline mutations), Cowden's disease (i.e., Phosphatase And Tensin Homolog (PTEN) germline mutations), APC germline mutations, and others.

Thyroid benign and malignant nodules are classified based in OMS histological criteria, that have been recently revised (REF) and that will be briefly described bellow.

1.4.1. Follicular Adenoma

Follicular adenomas (FA) are benign neoplasms that arise in the thyroid or in ectopic thyroid tissue, i.e. in tissue outside the usual topography of the thyroid, such as the neck or lateral cervical region (13). They are typically solitary nodules, encapsulated that can be in association with thyroiditis or nodular hyperplasia. Macroscopically FA have a brownish colour, solid and fleshy appearance. Histologically, they have a follicular architecture, with micro- or macrofollicular growth and normal cytology, with no evidence of invasion of the adjacent capsule or neighbouring tissue (15). Molecularly, genes from the RAS familly, H-, K-, N-RAS Proto-Oncogenes, may be involved in 20-30% of these tumours (16). *PAX8-PPARγ* rearrangement are found in 4-13% of follicular adenomas. The paired box 8 (*PAX8*) gene encodes a nuclear thyroid transcription factor involved in follicular cell differentiation. When PAX8 is rearranged, there will be a fusion with PPARγ impairing its function, consequently leading to loss of growth inhibitory controls. Hyperfunctioning adenomas, are a rare form of thyroid adenoma, resulting from a monoclonal expansion of follicular cells, with activating mutations in the TSH receptor gene or, less frequently, the G protein gene.

1.5. Thyroid Cancer

Thyroid cancer (TC) is the most frequent endocrine neoplasm, being the tenth most prevalent cancer in both sexes. It has a very low mortality, as it is generally a treatable cancer with a good survival for patients (17). Several factors are used to classify the various types of carcinomas, ranging from cell type, degree of differentiation and their architecture (18). This type of cancer is derived from follicular cells in 95% of the cases and is mainly classified into five histological types, namely papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), oncocytic carcinoma (OCA), poorly differentiated thyroid carcinoma (PDTC) and anaplastic thyroid carcinoma (ATC) (Table 1). PTC and FTC can be grouped into the differentiated thyroid carcinoma (DTC) group, which in turn accounts for most thyroid cancer cases. PDTC and ATC are less differentiated carcinoma (19).On the other hand, thyroid cancer can also originate from C cells, in which case it will give rise to medullary thyroid carcinoma (MTC) (Table 1).

Table1. Origin and subtypes of thyroid cancers.

Thyroid Cancer	
Follicular Cells	**C Cells**
Papillary Thyroid Carcinoma	Medullary Thyroid Carcinoma
Follicular Thyroid Carcinoma	
Poorly Differentiated Thyroid Carcinoma	
Anaplastic Thyroid Carcinoma	

1.5.1. Papillary Thyroid Carcinoma

Papillary thyroid carcinoma (PTC) is one of the most common and least aggressive histological types of all thyroid carcinomas (20) and originates from the follicular cells of the gland (21); it has a survival rate of more than 95%. PTC is an invasive neoplasm with ill-defined margins with whitish tones. It may contain calcifications, named as psammoma bodies. In terms of size, this histological type can variate in size from sub-centimetric lesions to large tumours, with an average diameter of two to three centimetres. Regarding the molecular pathogenesis of this carcinoma, we can see that activation of the Mitogen-Activated Protein Kinase (MAPK) signalling pathway plays a key role in carcinogenesis in thyroid carcinomas. Receptor tyrosine kinase (RTKs), RAS, RAF and other proteins are essential for this pathway, which in turn will lead to proliferation, differentiation and cell survival events (22).

Consulting the cBioportal database we realise that in PTC, 83% of the cases present mutations in the MAPK pathway. The most common alterations are in B-Raf Proto-Oncogene, Serine/Threonine Kinase (*BRAF*), namely the V600E mutation, in 62% of the cases, mutations in RAS, namely *KRAS*, *NRAS* and *HRAS* in 13% of the cases, *RET/PTC* rearrangement in 7% of the cases and, with a lower frequency, fusion in ALK Receptor Tyrosine Kinase (*ALK*), 0.85% of the mutated cases (23). Mutation in exon 15 of the BRAF gene (BRAFV600E) are the most common in patients with PTC. Previous studies have associated this mutation with a good prognosis for this histological type (24, 25). The *RAS* gene is part of the family of GTP-binding proteins, which regulate cell growth through two pathways, MAPK and PI3K-AKT. Mutations in *RAS* are described in all histological types of thyroid carcinoma, however in PTC the most frequent are in the *NRAS* gene, then in *HRAS* and infrequently in *KRAS*. These mutations are reported to alter GTP binding affinity or internal GTPase activity. In PTC, concomitant genetic alterations in *RAS* and in *BRAF gene*,are infrequent, indicating a common capacity to activate MAPK pathway between mutations in *RAS* and *BRAF* (26).

1.5.2. Follicular Thyroid Carcinoma

Follicular thyroid carcinoma (FTC) is the second most frequent histological type of DTC, comprising about 6% to 10% of all the thyroid cancers. A low iodine diet is presented as a risk factor, since it is more frequent in iodine deficient regions. FTC can be divided into three subtypes according to the World Health Organization (WHO) (27), into minimally invasive follicular carcinoma, characterised only by capsular invasion and usually has a good prognosis. Encapsulated angioinvasive carcinoma, charaterized by the presence of vascular invasion . Finally, there is the widely invasive follicular carcinoma, which involves extensive invasion of the capsule and can also invade the extrathyroidal tissue. This carcinoma is considered to have poorer prognosis in comparison with PTC, due to the higher frequency of haematogenous spread and distant metastases (17, 27).

Both in PTC and in FTC treatment requires a partial or almost total thyroidectomy, eventually complemented with radioiodine therapy. Thyroglobulin levels should be measured and periodic cervical ultrasound should be performed to detect recurrence (28).

1.5.3. Poorly Differentiated Thyroid Carcinoma

PDTC is a less common type of follicular cell carcinoma, with a rate of 1 to 3% of diagnosed cases. It mostly affects people in their 50's and is more prevalent in men. The prognosis of this carcinoma is intermediate,between DTC and ATC, with a mortality rate between 38% and 57% (29). Molecularly, mutations in RAS are more frequent in this carcinoma, between 20%–50%, but mutations in the TERT promoter (TERTp) are also very frequent, between 20%–50%, and a marker of poor prognosis for PDTC. It is an aggressive tumour with few potential therapeutic targets and a very low response to iodine therapy (30).

1.5.4. Anaplastic Thyroid Carcinoma

Anaplastic thyroid carcinoma (ATC) is rare but has a very high mortality and survival of less than 5%. They are fast-growing, infiltrating cervical structures such as the oesophagus, trachea or blood vessels and have the power to metastasise to the lung, bone and brain. This histological subtype can arise from differentiated thyroid carcinoma (CDT) but can also arise *de novo*. Even with therapy such as surgery, radiotherapy and chemotherapy, the average overall survival (OS) is only three to five months **(31)**. These tumours present a great heterogeneity between spindle cell, epithelioid and pleomorphic giant cells, being a huge challenge for pathologists to diagnose, however the most common histological type is spindle cell. Mutations in Tumor Protein P53 (*TP53*) are common in this carcinoma, as well as mutations in Telomerase Reverse Transcriptase promoter (*TERTp*) (32, 33).

1.5.5. Medullary Thyroid Carcinoma

Medullary thyroid carcinoma (MCT) is a rare subtype of thyroid carcinomas and accounts for 1% to 3% of all thyroid carcinomas, however it has a higher mortality rate than DTC. MTCs are mostly sporadic (75%) but can also be hereditary (25%) and associated with syndromes such as MEN2. Surgery, total thyroidectomy, is the only curative treatment, which can be combined with lymphadenectomy due to the frequent cervical lymph node metastases presence. Following surgery, radioactive iodine treatment is not adequate as MCT cells cannot capture iodine, being the new target treatments, such as tyrosine kinase inhibitors, the most common therapeutical approach (34).

1.6. *RRAS2* Gene

The Ras protein superfamily comprises more than 150 small GTPases subdivided into 5 main families according to their homology. Of these, the Ras related subfamily (*RRAS*) shares the highest sequence identity (52-55%) with the classical *HRAS*, *KRAS* and *NRAS*. RRAS incorporates *RRAS1*, *RRAS2* and *RRAS3* isoforms(35). Ras related protein 2 (*RRAS2*), better known as TC21, encodes the RRAS2 protein, which belongs to the RAS family of GTPases. These proteins play a key role in regulating intracellular signalling pathways that are involved in cell proliferation, migration, cell adhesion and angiogenesis. Structurally, RRAS2 has a similar structure to other Ras proteins, composed of a G (guanosine)-binding domain and a GTPase domain. These domains allow the RRAS2 protein to bind to guanosine diphosphate (GDP) or guanosine triphosphate (GTP) molecules, switching between an inactive (GDP-bound) and an active state (GTP-bound). When the RRAS2 protein is in the active form, it will interact with effector proteins and will trigger signalling pathways. These pathways include the activation of protein kinases such as protein kinase C (PKC), protein kinase B (Akt) and mitogen-activated protein kinase 3/extracellular signal-regulated kinase 1 (ERK). These signalling pathways have a key role in regulating cell growth, differentiation and survival (36). Mutations in this gene have been reported to be found in individuals with Noonan syndrome (NS), a rare disease that is associated with a dysregulation of the RAS signalling pathway (37). Members of the RRAS subfamily are involved in anchorage-independent growth, increased invasiveness, and stimulation of angiogenesis. They exhibit hyperactivation effects by promoting migration and invasion in some tumour types, whereas they inhibit the cell adhesion pathway. Mutations in *RRAS2* are rarely reported in human cancer, unlike mutations in classic RAS proteins. There are several tumours in which *RRAS2* is overexpressed, such as breast cancer, skin cancer, oesophageal cancer, oral cancer and central nervous system cancer (38).

1.7. Hotspot (Q72L/H) *RRAS2* Mutation

RRAS2 hotspot mutation, Q72L/H is a missense mutation, i.e. a change in a single amino acid at position 72 of the *RRAS2* gene, where glutamine (Q) is replaced by leucine (L) or histidine (H). This Gln72 is analogous to Gln61 which is found in RAS proteins, with the change in numbers being due to the presence of an additional N-terminal sequence of 11 amino acids in the primary structure of RRAS2. Mutations at position 72 are known to induce an oncogenic gain-of-function effect as they impair the hydrolysis of bound GTP and maintains these GTPases in the active state, leading to tumourigenesis (36, 39). A recent study investigated mutations in this hostpot in mouse models, hypothesizing that it could be a possible oncogenic driver in certain tumours. In the study, they used crispr for the mutation of the *RRAS2* hotspot and induced it in an animal model. From there, they went on to investigate whether there was any mechanism that could trigger tumourigenesis. The thyroid gland was not analysed in this study (40).

1.8. Telomerase , Shelterin Complex and *ACD* Gene

Telomeres are tandem arrangements made up of TTAGGG repetitive sequences at the terminal ends of chromosomes. These sequences play an important role in preserving genomic integrity, protecting, or covering the chromosome ends from degradation by DNA repair mechanisms and also preventing the loss of information during cell division. Over the course of divisions, telomeres get shorter and when they reach a critical limit, known as the Hayflick limit, the signalling pathways force entrance into senescence or apoptosis. There is a complex made up of six proteins that are involved in stabilising and maintaining the length and structure of telomeres (41), the shelterin complex. The proteins that compose the complex are telomeric repeat binding factor 1 (TRF1), telomeric repeat binding factor 2 (TRF2), repression/activation protein 1 (RAP1), nuclear factor interacting with TRF1 2 (TIN2), TIN2-POT1 organising protein (TPP1) and telomere protection 1 (POT1) (Figure 3). This complex presents a very complex architecture in which TRF1 and TRF2 have a structure that forms homodimers and that will bind to the DNA regions of the telomeres, TRF1 has the function of regulating the length of the telomeres by a process of replication of the telomeric DNA, while TRF2 will inhibi the recognition of the telomeres as DNA double-strand breaks (DSB). RAP1 will be recruited by interaction with TRF2, as this protein works with TRF2 to inhibit homologous recombination. POT1 will interact with TPP1 forming a stable heterodimer, they have functions together to block the ataxia telangiectasia and Rad3 (ATR) signalling pathway and regulation of telomerase activity. Finally, the binding protein is TIN2, which will link to TRF1, TRF2 and TPP1, facilitating the assembly of the shelterin complex (42, 43).

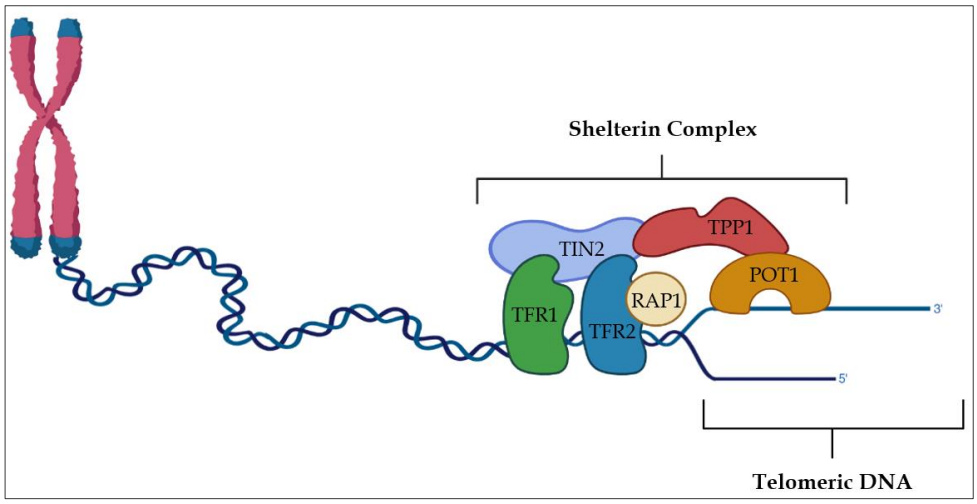


Figure 3. Representation of the structure of the shelterin complex. Telomeric repeat binding factor 1 (TRF1), telomeric repeat binding factor 2 (TRF2), repression/activation protein 1 (RAP1), nuclear factor interacting with TRF1 2 (TIN2), TIN2-POT1 organising protein (TPP1) and telomere protection 1 (POT1). Source: BioRender.com

Alterations in telomere biology are often caused by variants in genes associated with telomere elongation, structure, and function. One of those complex is telomerase, an enzyme that synthesises telomeric DNA sequences and that is frequently altereated in cancer. Its activity is absent in most somatic cells but is present (or reactivated) in more than 90% of cancer cells. This enzyme is made up of two components, a functional RNA (TERC), which serves as a template for synthesising telomeric DNA, and a catalytic protein (TERT) with reverse transcriptase activity (44, 45). In our model, thyroid tumours, mutations in the *TERT* promoter (TERTp) are common, present in all the histotypes, although more frequent in PDTC and ATC subtypes, as previously reported (46). Mutations in the *TERTp* play a fundamental role in the aggressiveness of thyroid carcinoma, leading to poorer clinical outcomes (47).

Adrenocortical dysplasia protein homolog (*ACD*), which encodes the TPP1 protein is another gene associated with diseases that modify telomere biology (48). TPP1 is one of the proteins that are part of the shelterin complex and is associated with several complexes with telomeric DNA. It plays an important role in the regulation of telomere length, as it recruits and activates telomerase (49). This protein is multidomain, i.e., it consists of a region that binds to telomerase and another region that binds proteins that are part of the shelterin complex. Structurally, TPP1 has an oligonucleotide/oligosaccharide binding fold domain (OB), a POT1-binding domain with a centred position (PBD) and a C-terminal TIN2-binding domain (TBD) (Figure 4). We can see that interactions between the complex and TERT are caused by the TPP1 and POT1 complete, since inhibitions and mutations in this subunit will give rise to a stable heterodimer that will increase telomere length, that is TPP1/POT1 is essential for inhibiting telomere lengthening (50).

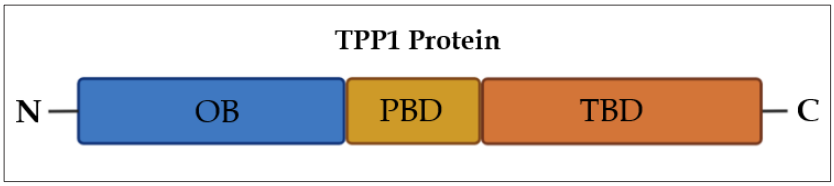


Figure 4. Structure of the protein TPP1. Oligonucleotide/oligosaccharide binding fold domain (OB) a POT1-binding domain with a centred position (PBD) and a C-terminal TIN2-binding domain (TBD)
Source: BioRender.com

Over the years, mutations in the TPP1 protein have been studied and reported in several diseases, notably dyskeratosis congenita (DC) and recently in cutaneous melanoma (51). Regarding the current study in melanoma, it has been reported that mutations in the TPP1 promoter (*TPP1p*) create new transcription factor binding sites, as reported for the TERT promoter (*TERTp*). Co-expression of these two mutated promoters leads to telomere lengthening, indicating that mutations in these promoters cooperate with cancer cell immortalisation. In addition, *TPP1p* was found to have two isoforms but only one expresses the variants that are associated with *TPP1p*, namely the -75 bp variant and the -108 bp variant with the C>T substitution. It is unknown if *ACD* mutations are present in thyroid tumors and it they can cooperate with TERTp in the telomere maintenance in cancer (52).

2. Objective

Taking into account that thyroid cancer ethiopathogenesis is characterized by oncogenic alterations leading to MAPK activation, and that advanced thyroid cancer presents often telomerase activation, we hypothesized that RRAS2 and/or ACD genetic alterations could be involved in thyroid cancer. Using molecular biology techniques, our aim was to assess the presence of mutations in the *RRAS2* gene and in the promoter of the *ACD*, in a large series of thyroid tumours.

3. Methods and Methodology

3.1. *In silico* Analysis

We analysed the two genes *in silico*, using computer tools and databases as cBioPortal, UniPROT, OncoMX, BioMuta, Genome-Tissue Expression (GTEX) and The Human Protein Atlas (THPA) . Initially, we extracted information from the genes for the functional and protein part based on UniPROT. We then used the GTEX portal to obtain the basal expression of these genes in normal tissues and OncoMx for biomarkers. BioMuta, was used to investigate mutations associated with *RRAS2* and *ACD*. It is important to refer that in the absence of databases characterizing the promoter region of *TPP1*, we evaluated the expression of the TPP1 mRNA coded by *ACD* gene. Both transcriptomic and genomic data were collected deposited data in cBioPortal from the Cancer Genome Atlas (TCGA). Regarding this database, for the study of thyroid tumours, a set of 633 samples from 625 patients are present in two studies on cBioPortal, referred to as "Thyroid TCGA Firehose legacy" (53) and "MSKCC, JCI 2016" (54).

3.2. Samples

For the genotyping, samples were collected at the Centro Hospitalar e Universitário de São João (CHUSJ) in the Pathology Department. The DNA samples used were available at the Cancer Signalling and Metabolism biobank present in i3S. For quantity determination (ng/µl) a spectrophotometer NanoDrop One (Thermo Scientific, MA, USA), was used and quality of nucleic acids was estimated from the 260/280 and 260/230 ratios.

All the necessary information on the series used to develop the project was provided by the Pathology Department of the Centro Hospitalar e Universitário de São João, based on pathology reports. The information provided ranged from the histological type and subtype of the tumour, patient data such as age and gender, information on the tumour, from its size, the number of existing tumours, the presence or absence of a tumour capsule and presence or absence of invasion, but also vascular invasion, presence of metastases, extra-thyroid invasion and the presence and type of infiltrate. According to the availability of suitable tissue, pathological report and/or clinical information, 179 samples from 173 patients were selected for the subsequent *RRAS2* gene study and 110 samples from 94 patients for the *TPP1p* study. These included both benign and malignant lesions. For the *RRAS2*, we evaluated 63 follicular adenomas; 81 papillary thyroid carcinoma (PTC); 14 folicullar thyroid carcinoma (FTC); 8 medullary thyroid carcinoma (MTC); 5 poorly differentiated thyroid carcinoma (PDTC) and 2 oncocytic carcinoma (OC). On the other hand, for *TPP1p*, 81 PTC; 14 FTC; 8 MTC; 5 PDTC and 2 OCA.

This study complies with the Declaration of Helsinki and the protocol was approved by the CHUSJ Ethics Committee. The samples were anonymised and the study was retrospective which excepts patient consent to the study, in accordance with national ethical guidelines.

3.3. Primers and Optimization

The primers for exon 1 of the *RRAS2* gene hotspot and for the *TPP1p* were designed *in-house*; the sequences are represented in table 2 and 3, respectively. Working solutions of primers for Polymerase Chain Reaction (PCR) were prepared at a concentration of 10 µM. Primer temperature optimization was done using a temperature gradient (from 56ºC to 64ºC) for best annealing and product amplification performance (Figure 5). The amplicons of PCR temperature gradient were subjected to agarose gel electrophoresis to select the conditions that obtain more specific fragments and with greater efficiency.

Table 2. Design of forward and reverse primers sequences for the *RRAS2* gene hotspot.

Primer	Sequence (5'->3')	Length	TM (ºC)	GC (%)
Forward	CCAGGAGAAGTACCGGCTCG	20	66	62
Reverse	GCCACAGGTAGCGCCAGCC	19	66	74

Table 3. Design of forward and reverse sequences for the TPP1 promoter (TPP1p) of the *ACD* gene.

Primer	Sequence (5'->3')	Length	TM (ºC)	GC (%)
Forward	CCAGGAGAAGTACCGGCTCG	20	66	62
Reverse	GCCACAGGTAGCGCCAGCC	19	66	74

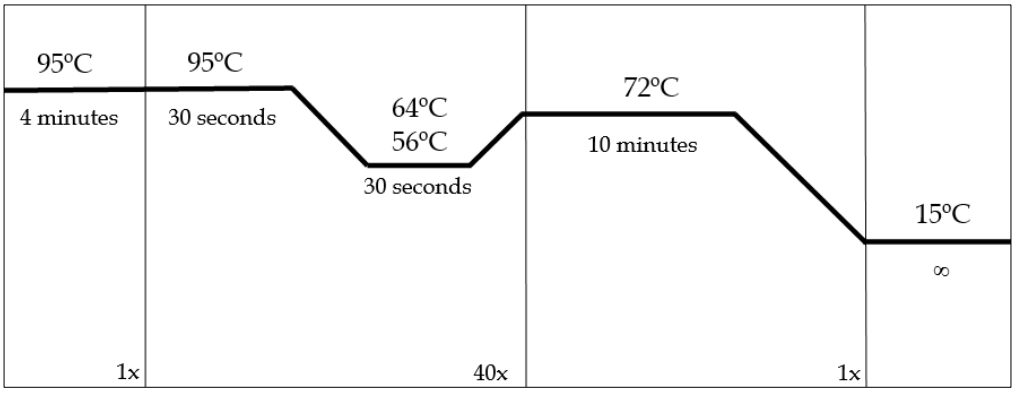


Figure 5. Programme used to find the best temperature of the primers through a gradient of temperatures.

3.4. Amplification by PCR

To carry out the PCR technique, a series of steps were used, namely the preparation of a mix consisting of 5 µl of MyTaq HS Mix 2x (Bioline, London, UK), 0.5 µl of both forward and reverse primer mix in the same mix and 3.5 µl of water, at the end of the process we added 1µl of DNA from the respective sample under study. The second stage is carried out in a thermal cycler using the following cycling conditions, Figure 6 .

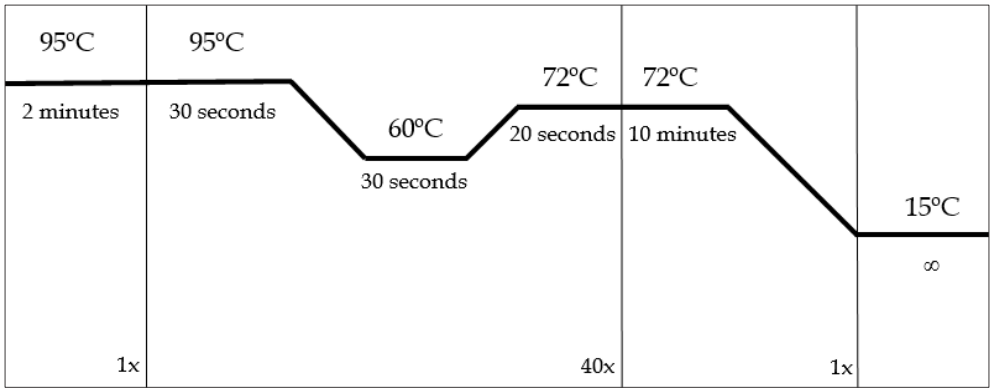


Figure 6. Programme for Polymerase Chain Reaction (PCR).

3.5. Agarose Gel Electrophoresis

Once the PCR finished amplification, we evaluated wheter the respective amplicons were present by visualization in an agarose gel electrophoresis. For this step, we made the base of the 1% agarose gel, containing 0.50 g of agarose in 50 ml of SGBT buffer (GB01.0120, GRISP, Porto, Portugal) and let it polimerize. We then used a mixture of bromophenol blue to give colour to the amplified fragments and a DNA intercalating agent, RedSafe (iNtRON Biotechnology, WA, USA) to provide fluorescence. Place a drop of this mixture on a parafilm base and add 2 µl of the PCR product for each sample. Finally, we pipetted the solution with the DNA onto the solidified agarose base and run it at 180 Volts. The final image of the fragments was obtained on GelRed (Bio-Rad, CA, USA).

3.6. Enzyme Purification

For Sanger sequencing, each amplified product was purified with 1.5 µl of ExoSap enzyme mixture, containing 0.5 µl of Exonuclease I (Exo) (20U/µl, EN0581, ThermoFischer) and 1 µl of Shrimp Alkaline Phosphatase (SAP) (ThermoFischer); these enzymes aimed to degrade remainig components of the PCR and cleave the phosphate groups. For the activation and activity of the enzymes, they were placed at 37ºC for 30 minutes and a final denaturation step of 85ºC for 15 minutes, in a single cycle in the thermal cycler.

3.7. Sequencing PCR

The sequencing reaction generates DNA strands with different sizes terminated with dideoxynucleotides (ddNTPs) with different fluorochromes. Each gene was sequenced independently with the corresponding primer. For each sequencing reaction, 1 µl of ExoSap cleaned-up product was mixed with 3.4 µl of sequencing buffer, 3.5 µl of DNAse water, 0.3 µl of reverse or forward primer and 0.25

µl of BigDye enzyme (Table 4). For the Sequencing PCR reaction, the following cycle conditions were used, presented in Figure 7.

Table 4. Quantities required for the sequencing PCR reaction.

Mix	Volume (1x) (µl)
H20	3,5
Buffer	3,4
Primer	0,3
Bigdye	0,4

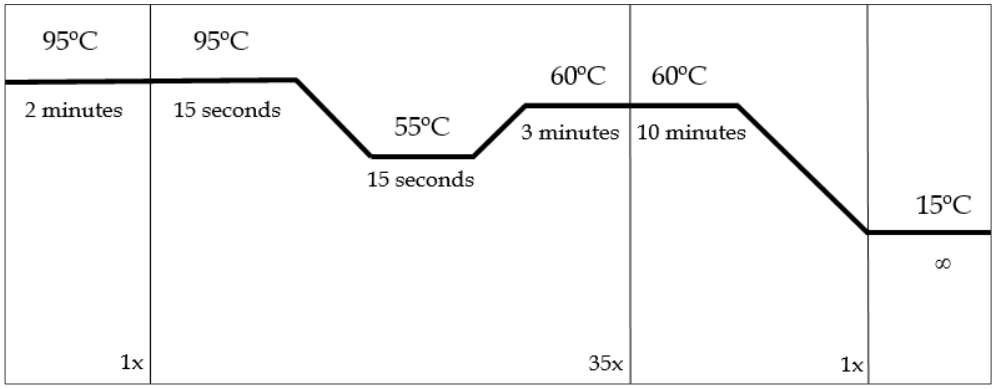


Figure 7. Programme for sequencing PCR reaction.

3.8. Sephadex Purification and Formamide

Post-sequencing purification was carried out using a Sephadex solution (G50150, GE Healthcare, IL, USA). Each sample had a tube containing 750 µl of Sephadex and was centrifuged at 3400 rpm for 4 minutes. After this purification, 15 µl of hi-di formamide (4311320, Applied Biosystem) was added to chemically denature the DNA for linearization. The final product was analysed on the ABI3130 Genetic Analyser (Applied Biosystems).

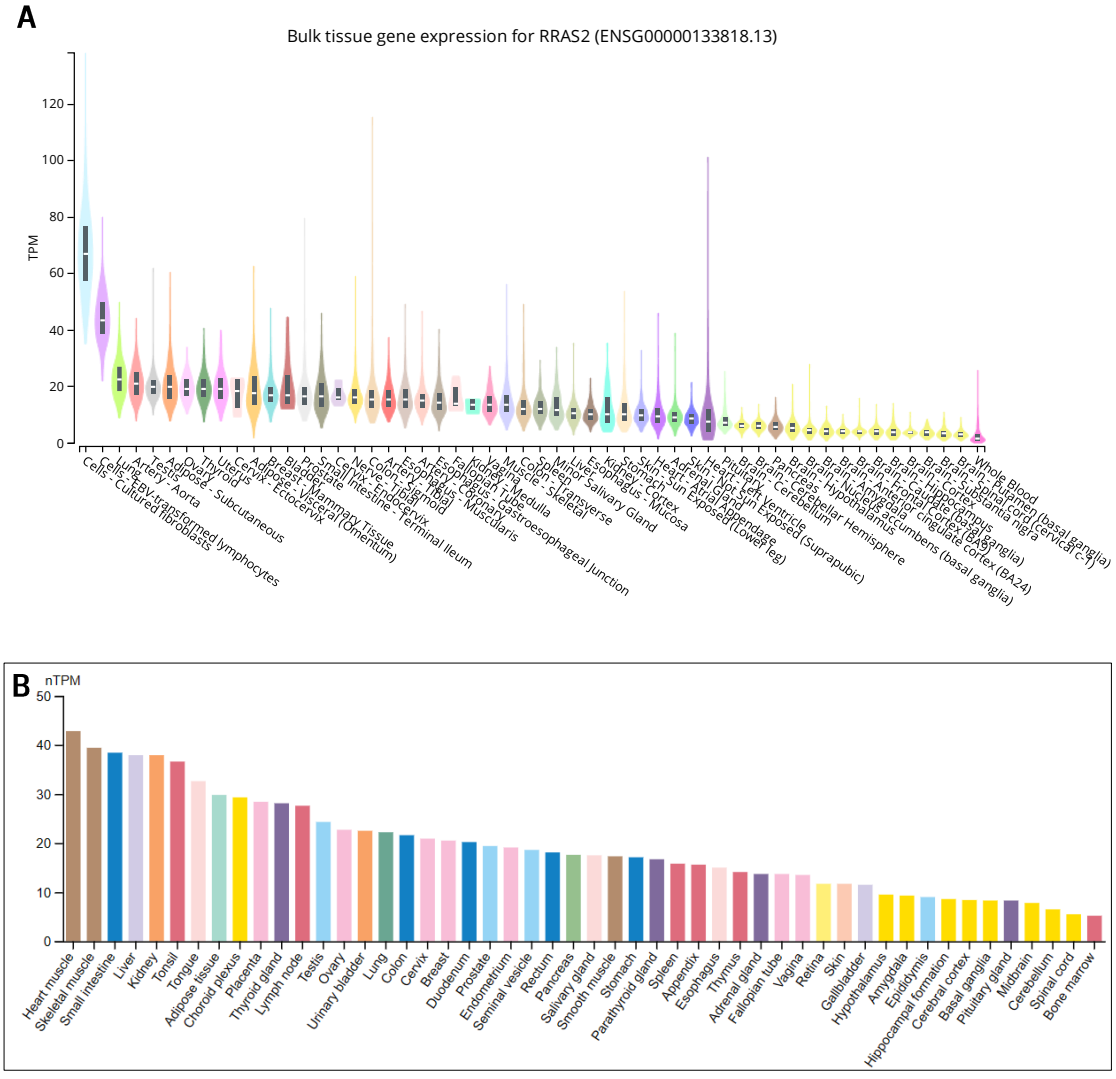
3.9. Statistical Analysis

Representative Statistical analysis was conducted with IBM® SPSS® Statistics for Mac, version 24.0 (IBM, Chicago, USA). The results are expressed as a percentage. Figures were done in GraphPad Prism v9.0. for Mac.

4. Results

4.1. *In silico* analysis of *RRAS2*

Using the GTEx portal (55), which gives us the expression in normal tissues, we observed that the lung is the organ with the highest expression of *RRAS2*, with 22.39 transcripts per million (TPM), while the thyroid is the fifth organ with the highest expression with 19.12 TPM, Figure 8A. Using The Human Protein Atlas (THPA) (56), we analysed RNA expression in the gene under study and found that the thyroid is the fourth organ with the most RNA expression, with 28.1 normalised transcripts per million (nTPM), Figure 8B. Analysing RRAS expression in normal tissues using The Human Protein Atlas (THPA), protein expression is classified as medium and the RRAS2 protein has an intracellular localisation, Figure 8C.



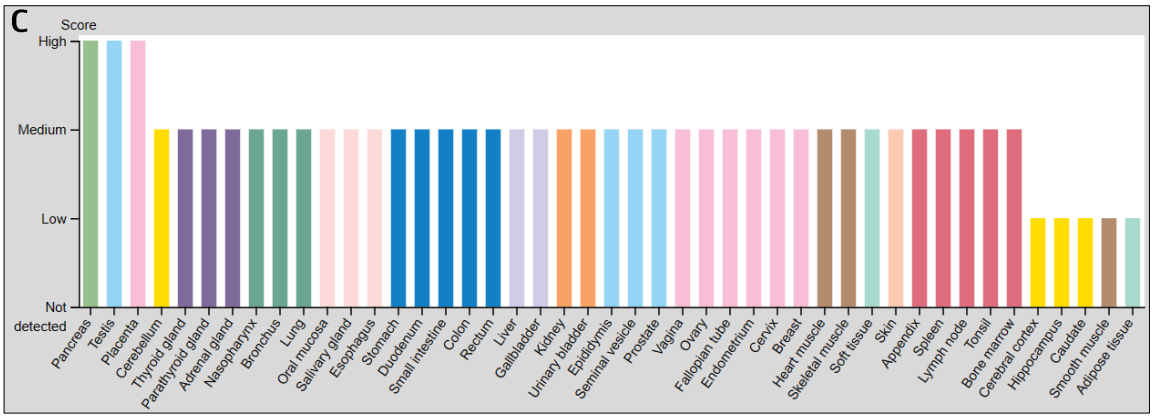
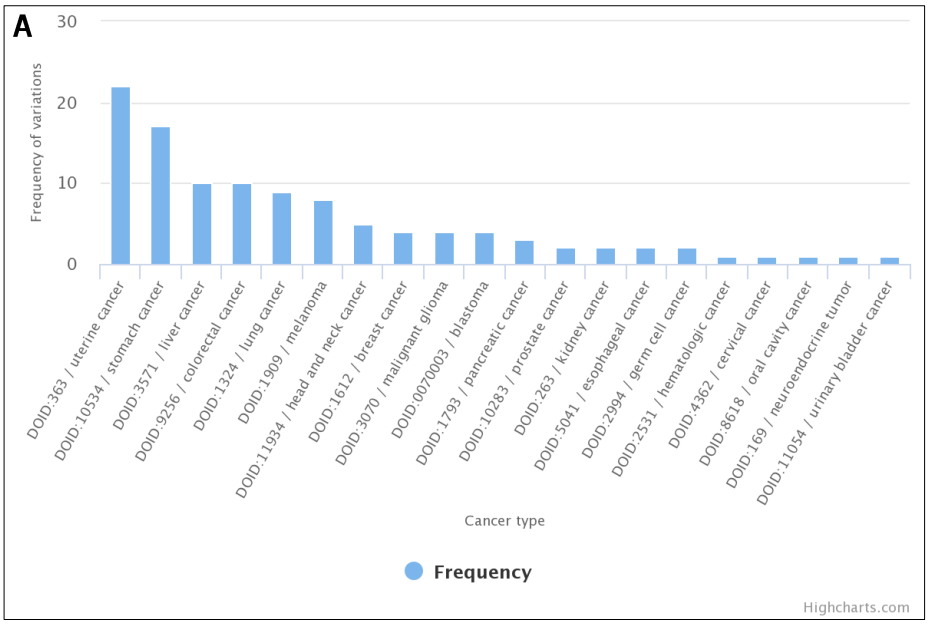
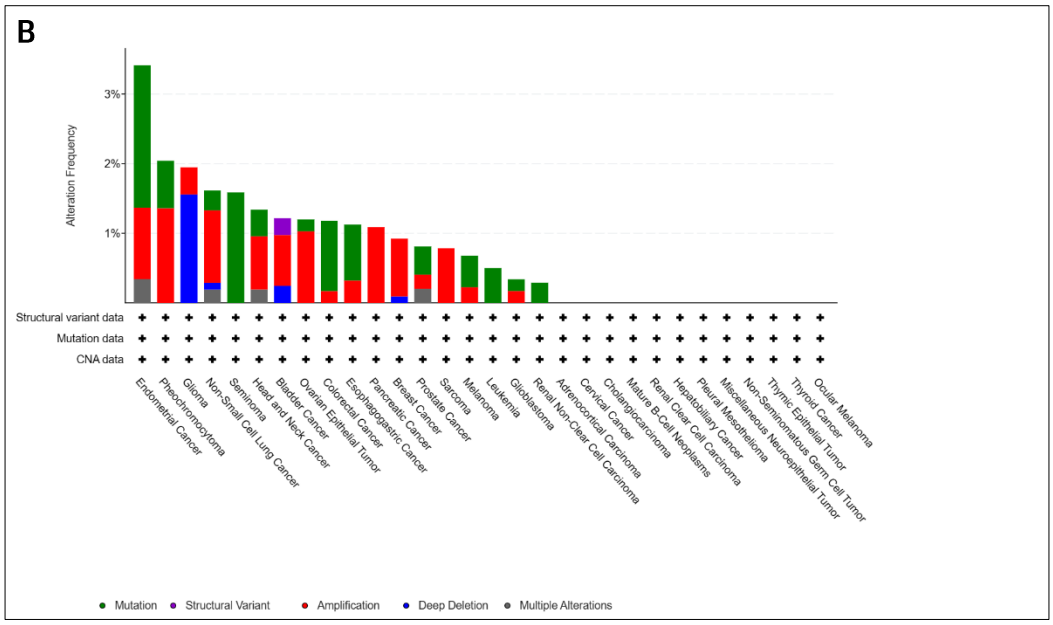


Figure 8. *RRAS2* characterization – an *in silico* approach. A: *RRAS2* gene normal tissue expression according to different human organs, data from GTEx; B: Transcriptomic overview of RRAS2 by tissue, Figure extracted from The Human Protein Atlas (THPA); C: RRAS2 protein expression in different tissues, Figure extracted from THPA.

From the BioMuta database (57), we identify several mutations for *RRAS2*, particularly in uterine cancer, but for thyroid no single nucleotide variant (SNV) was detected, Figure 9A. On the other hand, to get a better overview of existing *RRAS2* mutations, we used cBioPortal (58) to extract data from 10967 samples from 10953 patients with the TCGA PanCancer Atlas Studies filter. In a first analysis, we focused on the various types of existing alterations for the candidate gene, and we observed the presence of missense mutations, amplifications, deep deletions and structural variants, which are variations in the number of copies that can be inversions, deletions or insertions, in various types of cancers, Figure 9B. In Figure 9B, we also highlight the fact that there were no mutations in thyroid carcinoma, whereas endometrial carcinoma has a higher frequency of alterations in *RRAS2*. Of the 10,967 cases, 48 mutations were recorded. Of all the cases analysed, 13 cases were recorded with the Q72L/H mutation, the most frequently alteration along the gene, Figure 9C, mainly in uterine cancer and in non-small cell lung cancer.





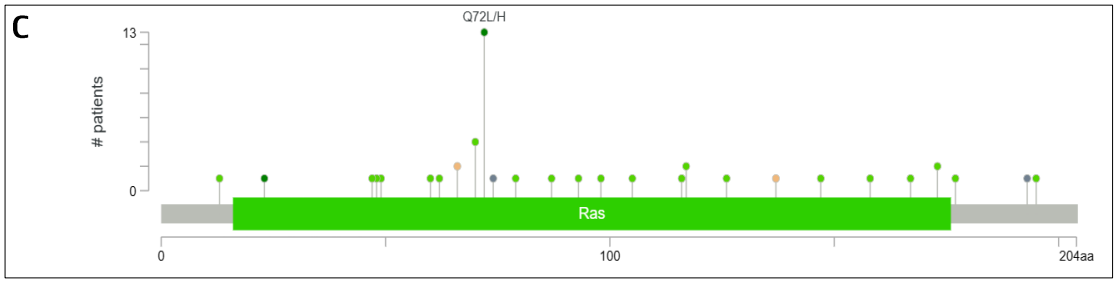


Figure 9. A PanCancer overview of *RRAS2* in cancer. A: Demonstration of several cancers with alterations in the *RRAS2* gene. B: *RRAS2* alterations frequency in the 10967 samples of 10953 cases from TCGA PanCancer Atlas Studies; C: Distribution of the genetic alterations (spots) detected throughout *RRAS2* gene. Figures extracted from BioMuta and cBioPortal for Cancer Genomics.

To perform the same analysis as above, but now centred in thyroid cancer, data were extracted from cBioPortal (58) which includes the sequencing studies of poorly differentiated and anaplastic thyroid cancers (MSKCC, JCI 2016) (54) and thyroid carcinoma (TCGA, Firehose Legacy) (53). We extracted 633 cases from 625 patients and concluded that there were no mutations reported for this specific cancer, Figure 10.

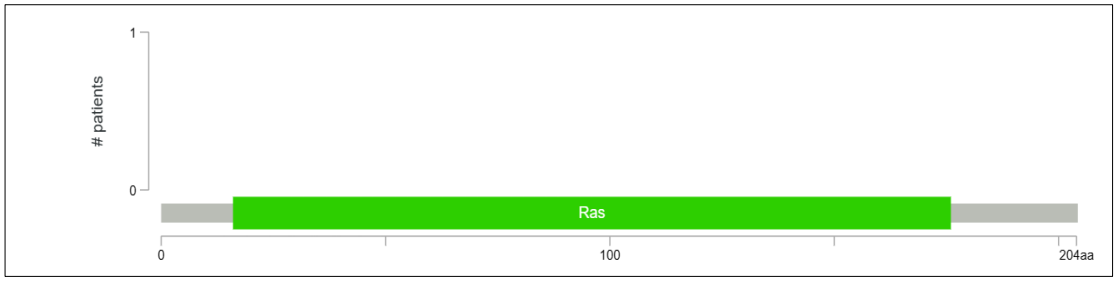
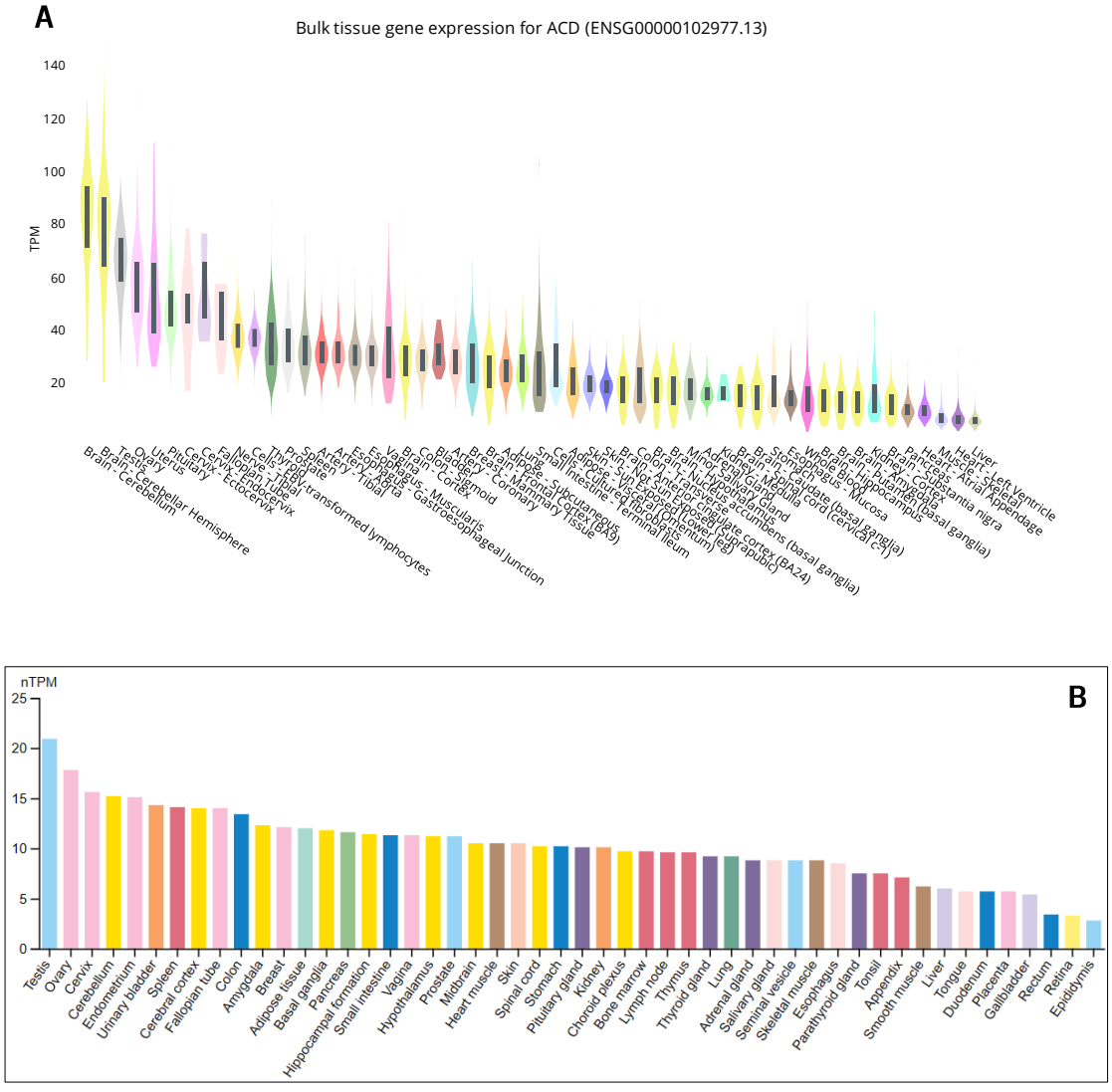


Figure 10. *RRAS2* molecular profiling in thyroid carcinoma. Distribution of the 0 mutations.

4.2. *In silico* analysis of *ACD*

The *ACD* gene is located at 16q22.1 and has 12 transcripts that produce 2 isoforms obtained by alternative splicing (59, 60). Since the study is in the *ACD* promoter zone, but we were unable to obtain the non-coding region of the gene *in silico*, we opted to do an *in silico* analysis of the coding region in order to have information on *ACD* expression. From the GTEx portal (61) for normal tissues, we found that the brain is the organ with the highest expression of *ACD*, with 85.20 transcripts per million (TPM), while the thyroid is the fourth organ with the highest expression with 33.89 TPM, Figure 11A. After analysing its expression in normal tissues using The Human Protein Atlas (THPA) (62), we analysed RNA expression in the gene under study and found that the thyroid is one of the organs with the lowest RNA expression, with 9.2 normalised transcripts per million (nTPM), Figure 11B. On the other hand, protein expression is classified as medium and the TPP1 protein has an intracellular localisation, Figure 11C.



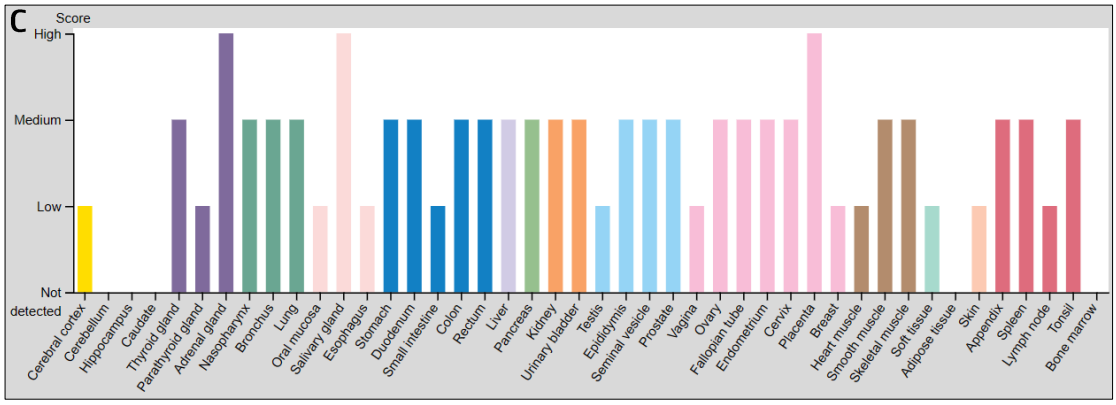
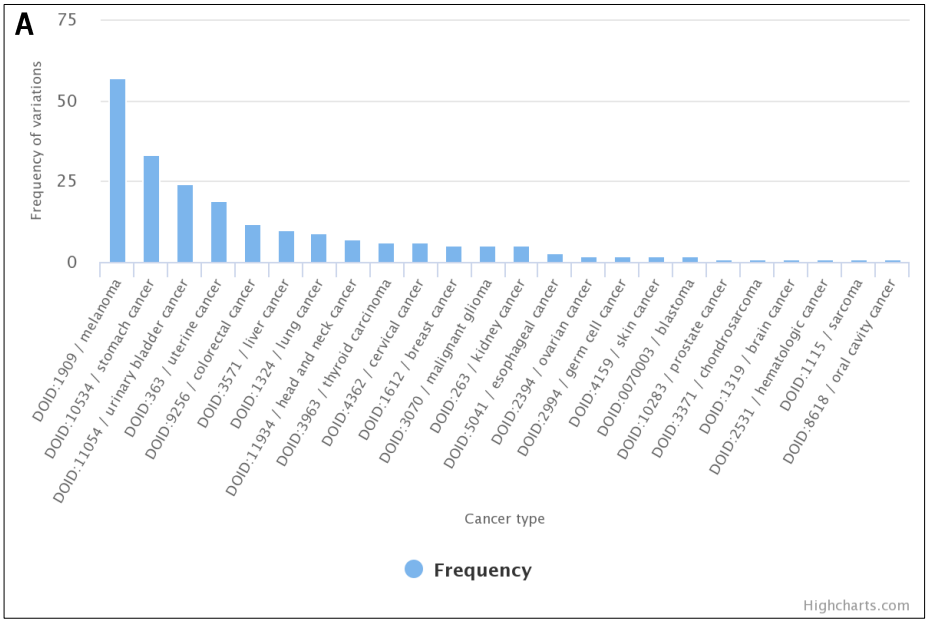
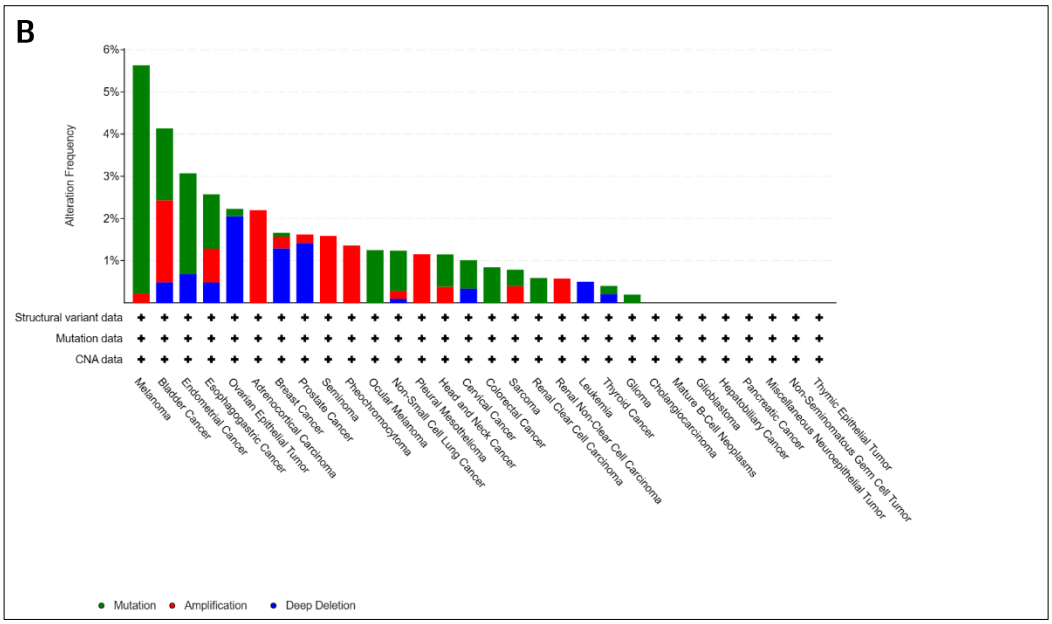


Figure 11. *ACD* characterization – an *in silico* approach. A: *ACD* gene normal tissue expression according to different human organs, data from GTEx; B: Transcriptomic overview of *ACD* by tissue, Figure extracted from THPA; C: TPP1 protein expression in different tissues, Figure extracted from THPA.

From the BioMuta database (63), we report a huge frequency of alterations for *ACD*, particularly for melanoma, but for thyroid their frequency is low, Figure 12A, and no single nucleotide variant (SNV) was detected. On the other hand, to get a better overview of the existing mutations in *ACD*, we used cBioPortal (64) where we extracted data from 10967 samples from 10953 patients with the TCGA PanCancer Atlas Studies filter. In a first analysis, we focused on the various types of existing alterations for the gene under study, and found that various types of cancers present missense mutations, amplifications, deep deletions, Figure 12B. In Figure 12B, we can also emphasise the fact that there is a low frequency of alterations for thyroid carcinoma, which contrasts with the case for melanoma, which has a high frequency of alterations in *ACD*. Of the 10967 cases, 88 mutations were recorded and the most frequent mutation is L62F/I with 5 mutated cases, Figure 12C.





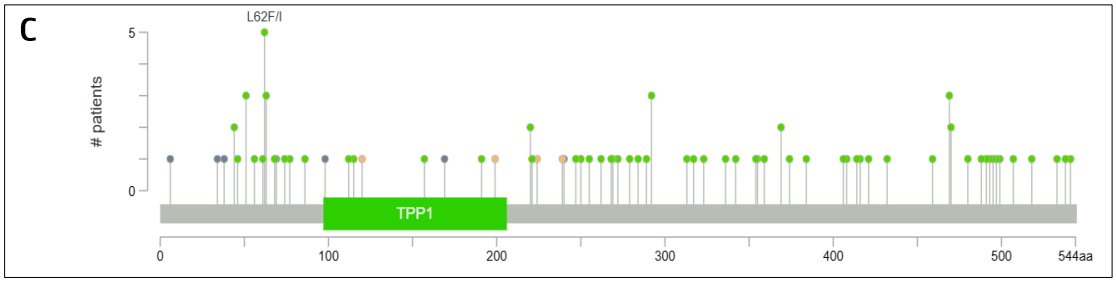
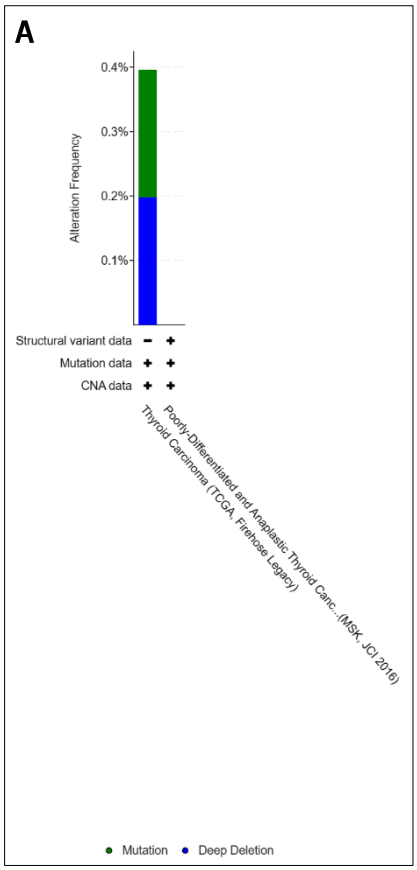


Figure 12. A PanCancer overview of *ACD* in cancer. A: Demonstration of several cancers with alterations in the *ACD* gene. B: *ACD* alterations frequency in the 10967 samples of 10953 cases from TCGA PanCancer Atlas Studies; C: Distribution of the genetic alterations (spots) detected in *ACD* gene throughout *ACD* gene; Figures extracted from BioMuta and cBioPortal for Cancer Genomics.

To perform the same strategic analysis as above for thyroid cancer, data were extracted from cBioPortal (64) that include the sequencing studies of poorly differentiated and anaplastic thyroid cancers (MSKCC, JCI 2016) (54) and thyroid carcinoma (TCGA, Firehose Legacy) (53). A total of 633 cases from 625 patients were extracted and best fit data for deep deletion and missense mutations are available for well-differentiated thyroid carcinoma studies, as depicted in Figure 13A. Only one mutation was identified: p.H292R, in 1 case of PTC (Figure 13B). Again, no mutation was reported for the promoter site of this gene in this study.



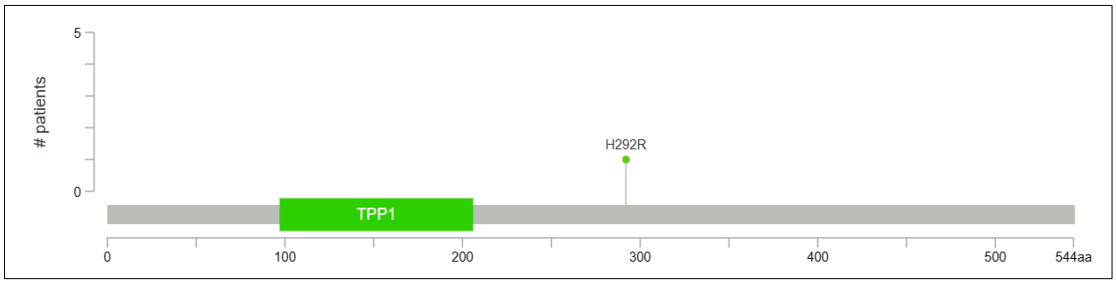


Figure 13. *ACD* molecular profiling in thyroid carcinoma. A: Alteration frequency in Thyroid Carcinoma (TCGA, Firehose Legacy) and Poorly Differentiated and Anaplastic Thyroid Cancers (MSKCC, JCI 2016); B: Distribution of the one mutation. Figures extracted from cBioPortal.

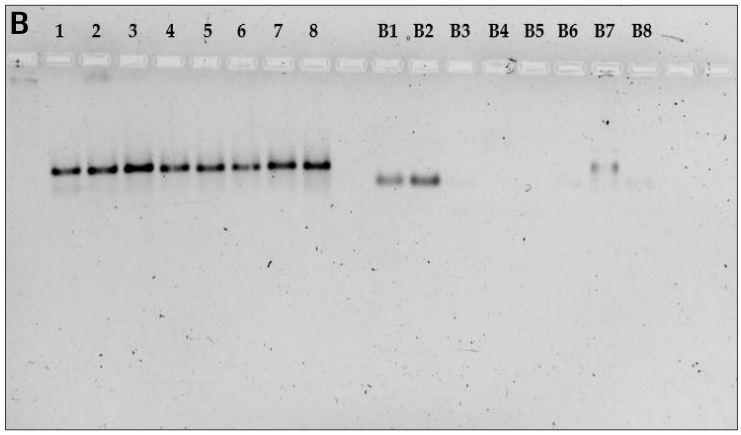
4.3. Optimization of primers *RRAS2* and *TPP1 promoter*

Following *in silico* analysis of the genes, we started optimising the amplification with the respective primers. Regarding the *RRAS2* gene, we proceeded with the existing protocol for optimisation, starting with the amplification PCR in which we tested temperatures between 56ºC and 64ºC , Figure 14A, and later an agarose gel electrophoresis to verify which of the temperatures amplified more efficiently the zone of interest (amplicons), Figure 14B. From the agarose gel analysis we proceeded to Sanger sequencing of each of the amplicons for the diferent temperatures. It was determined that the

temperatures of 58.9ºC and 60.8ºC were the ones that presented the best results, so we decided to use the intermediate temperature of 60ºC, Figure 14C.

A

Samples	Well ID	T(°C)
1	A	64
2	B	63,4
3	C	62,4
4	D	60,8
5	E	58,9
6	F	57,5
7	G	56,5
8	H	56



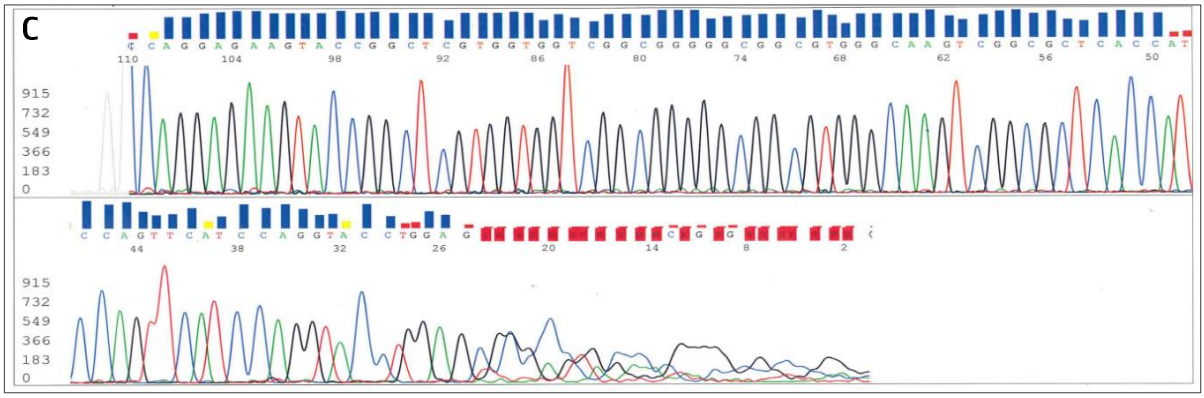


Figure 14. A: table with the temperatures in the PCR for the temperature gradient; B: confirmation of the amplification of the region of interest; C: chromatogram of the sequence with the chosen temperature of 60ºC.

For the *ACD/TPP1p* the same procedure was performed as in the *RRAS2*, and a temperature of 60ºC was selected. In Figure 15, is the corresponding agarose gel results and the sequences chromotagrams demonstrating the optimal temperature for this primer.

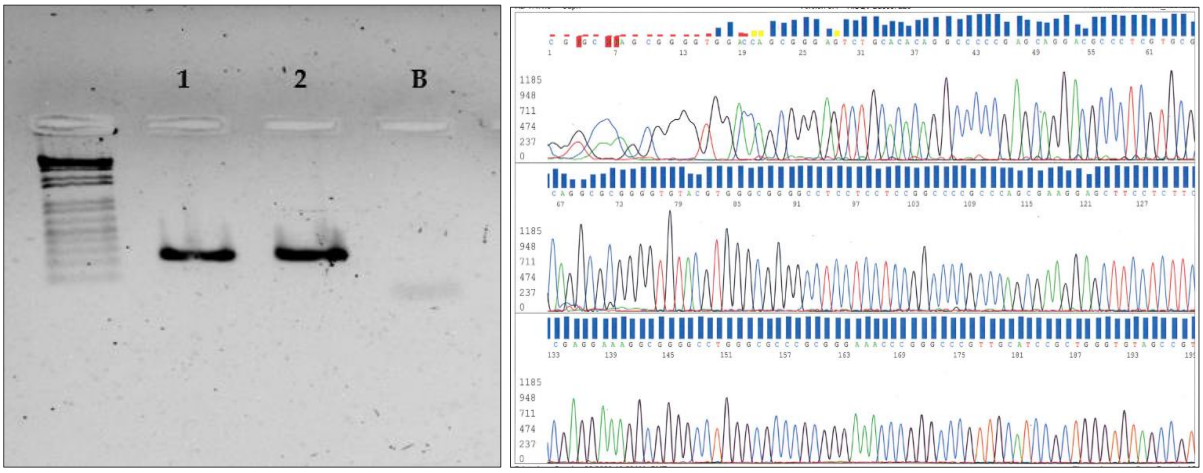


Figure 15. Demonstration of the amplification of the region of interest through the gel and the sequence with the optimal temperature.

4.4. *RRAS2* Mutation

A total of 179 samples from 173 patients were genotyped. The samples included 63 follicular adenomas, 81 PTC, 14 FTC, 8 MTC, 5 PDTC and 2 OC. Table 5 shows the clinicopathological parameters collected for the study cohort.

Table 5. Clinicopathological characterization of the series

		Histological Types					
		Adenomas (n=63)	**PTC** (n=81)	**FTC** (n=14)	**PDTC** (n=5)	**OC** (n=2)	**MTC** (n=8)
Age (mean)		42.5	44.5	45.1	43.1	44.7	47.7
Gender n (%)	Men	15(23.8)	17(21.0)	4(28.6)	3(60.0)	0(0.0)	5(62.5)
	Female	48(76.2)	64(79.0)	10(71.4)	2(40.0)	2(100,0)	3(37.5)
Tumour size (mean, mm)		3.2	3.0	3.0	2.9	2.5	2.7
Vascular invasion n (%)		NA	26/81 (32.1)	5/14 (35.7)	2/5 (40.0)	1/2 (50.0)	5/8 (62.5)
Tumor capsule n (%)		NA	31/81 (38.3)	13/14 (93.0)	1/5 (20.0)	1/2 (50.0)	3/8 (37.5)
Capsular invasion n (%)		NA	17/81 (21.0)	12/14 (85.7)	1/5 (20.0)	1/2 (50.0)	0/8 (0.0)
Lymph node metastasis n (%)		NA	22/81 (27.2)	1/14 (7.1)	2/5 (40.0)	0/2 (0.0)	4/8 (50.0)
Lymphocytic infiltrate n (%)		NA	29/81 (35.8)	3/14 (21.4)	0/5 (0.0)	0/2 (0.0)	1/8 (12.5)

NA: notapplicable; PTC: Papillary thyroid carcinoma; FTC: Follicular thyroid carcinoma; PDTC: Poorly differentiated thyroid carcinoma; OC: Oncocytic carcinoma; MTC: Medullary thyroid carcinoma.

The evaluation of the *RRas2* Q72L/H hotspot mutation was performed on all the series. No mutations were identified in the hotspot, with no substitutions of the glutamine protein for a leucine or histidine at position 72, Figure 16. The entire series was wild type for *RRAS2*.

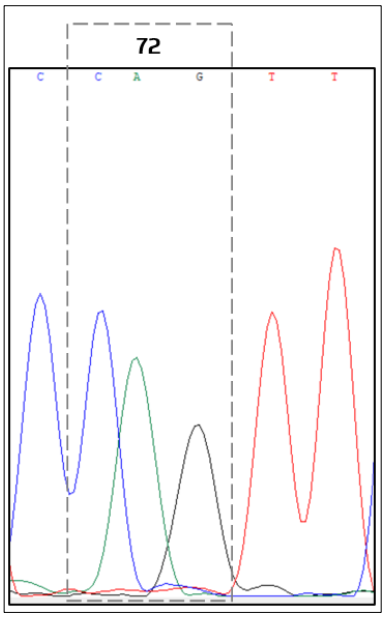


Figure 16. Chromatogram of the zone of interest of the Q72L/H hotspot.

4.5. *TPP1* Promoter Mutation

A total of 110 samples from 94 patients were genotyped. The samples included 81 PTC, 14 FTC, 8 MTC, 5 PDTC and 2 OC. The series under study was the same for the *RRAS2* genotyping, except for the follicular adenoma samples, since for the *TPP1p* the analysis was only carried out on carcinoma. No mutations were identified in the two *TPP1p* variants without the cytosine base replaced by a thymine either at position -75 bp or at position -108 bp, Figure 17. The entire series was wild type for *TPP1p* hotspot.

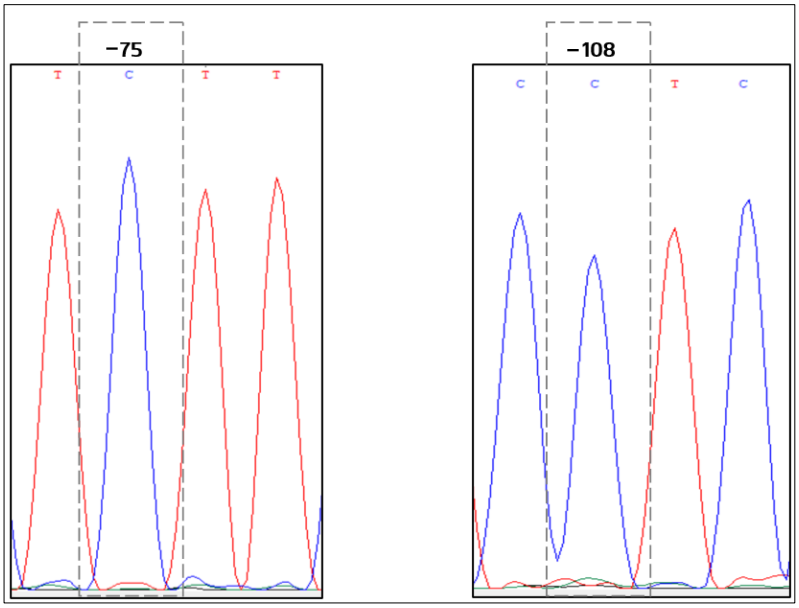


Figure 17. Chromatogram of the zones of interest of the TPP1 promoter.

5. Discussion

The *RRAS2* gene, located at 11p15.2, has 5 exons that encode the RRAS2 protein, which belongs to the RAS family of GTPase proteins that participate in the RAS-MAPK pathway. The similarity of this protein to the HRAS protein is more than 50 per cent and it consists of the same four functional domains as the main RAS proteins (37). It is involved in various cellular processes, such as proliferation, survival and migration, as well as functional deregulation, which are known to contribute to the process of tumourigenesis. Variants in this gene will affect its function, including nucleotide binding, GTP hydrolysis and effector interactions. On the other hand, it will play a major role in the MAPK pathway, potentially leading to increased activation and, in turn, deregulation of the MAPK cascade (67). This gene has not been intensively studied in cancer, but mutations in *RRAS2* have been reported to trigger tumor formation, particularly in the uterus and endometrium. With regard to the long-tailed hostpot (Q72L/H) under study, the literature tells us that studies are scarce, but from the little that does exist, mutations are described in a rare pathology called Noonan Syndrome (NS) syndrome also associated to other RAS mutations. Given the relevance of MAPK activation in cancer *RRAS2* mutations can also lead to tumourigenesis (39). Through in silico analysis, the data clearly shows that *RRAS2* is expressed in normal tissues of certain organs, such as the thyroid. On the other hand, when we analyze the data on the presence of variants, of the 10967 samples provided by cBioPortal (66), there were a few alterations, for certain organs and tissues reported. Specifically for the hotspot under study (Q72L/H), this hotspot was one of those with a higher mutation frequency for various organs, but for the thyroid the data did not record any Q72L/H mutations. With regard to the data from our series within the scope of this study, the databases are in agreement with our results, i.e. we did not report the presence of any missense mutation in the hotspot for 179 samples, both in benign and malignant tumours. Knowing that *RRAS2* belongs to the RAS family of GTPase proteins, the RRAS2 protein binds to GDP or GTP, alternating between the protein's inactive and active state, respectively, once it is in its activated form (bound to GTP) it will be able to interact with effector proteins and, in turn, trigger the normal functioning of signalling pathways (38). As already mentioned, *RRAS2* is one of the activators of the MAPK pathway, one of the most important pathways in thyroid tumourigenesis, so alterations targeting *RRAS2* were interesting candidates. With regard to the Q72L/H hotspot, it has been reported that mutations at this position induce a gain of function, causing defects in the hydrolysis of active GTP and triggering tumourigenesis. In a recent study, using animal models modified with the mutation in Q72L/H, the authors found that all the mice ended up dying due to tumour formation in certain organs after being treated with tamoxifen for gene activation. We know that *RRAS2* is important for activating the MAPK pathway and that this pathway is important for cell proliferation, survival and migration. Therefore, deregulation of this pathway ultimately leads to the formation of tumourigenesis (42). So, based on our analysis, we can conclude that what was reported in that article doesn't recapitulates biological activity in human tumours, at least in which concerns thyroid,

since we didn't report any mutation in the thyroid. However, it should be noted that this hotspot is not easy to sequence, as it has many GC repeats, which makes it difficult to design and optimize primers. Concerning a possible homology with the *RRAS2P1* pseudogene, we compared the sequences and found no homology between the two. As this is one of the reasons why mutations go unnoticed, it was important to characterise them.

The adrenocotical dysplasia homolog (*ADC*) gene encodes a telomere binding protein called TPP1. This protein has the function of telomere stability as well as regulating telomere growth (65). Telomeres are telomeric DNA repeats that are linked to a complex of six proteins called shelterin. This complex will protect the ends of the chromosomes from being recognised as double-stranded (ds) DNA breaks (66). Shelterin has functions such as protecting telomeres from degradation, aberrant recombination, inadequate processing by the DNA repair pathway, and facilitating chromosome capping to mediate telomerase activity. In this way, alterations in the proteins of the complex will lead to the origin of pathologies, most notably cancer (67). We can say that short telomeres will cause permanent DNA damage responses, which will consequently lead to cellular senescence. Therefore, the existence of stability in telomere lengths is fundamental for cell changes and the shelterin complex plays a crucial role in this stability (68). Since this gene is important for shelterin stability, we studied its promoter as it is the starting point for the gene's transcription process. With regard to the promoter, it was more complicated to carry out an *in silico* study because there were no bioinformatics databases with information on promoter regions. Our analysis therefore centered on the *ACD* gene, which produces the TPP1 protein, hence the name of the promoter, *TPP1p*. We found that *ACD* expression was high in normal tissues, but that RNA expression was low in thyroid tissues. As with *RRAS2*, protein expression was also average. A more deep analysis, using cBioPortal, reveal that mutations in this gene are not very common in the thyroid gland, with only one case having been reported in PDTC. In our series, *TPP1p* genotyping revealed that there were no mutations in the cases studied. TPP1 protein, one of the six proteins that make up the shelterin complex, plays a role in regulating telomere length, as it recruits and activates telomerase. Based on cBioPortal data on *TERT* mutations, in a study of 663 samples from 665 patients, we found that there is a high frequency of *TERTp* alterations in poorly differentiated and anaplastic thyroid carcinoma, compared to other histological subtypes such as PTC, FTC and MTC, with the latter subtype being almost absent. In conclusion, we were the first to evaluate *TPP1p* mutations in the thyroid. Unlike *TERTp* mutations, which are common in melanoma and thyroid carcinoma, *TPP1p* only seems to be associated with melanoma and is therefore an irrelevant alteration for the thyroid tumourigenic process.

6. Conclusion

We concluded that the data we obtained from genotyping the *RRAS* gene and the TPP1p is in line to what is present in certain bioinformatics databases. *RRAS2* does not present any type of oncogenic event for thyroid tumours and therefore does not trigger tumorigenesis, which contrasts to what was observed in a generated mousse model *in vivo*. Regarding the *TPP1* promoter, we found that mutations in this hotspot are a very rare event for thyroid tumours although we cannot exclude that for other tumour models it may represent a driver of tumorigenesis.

Bibliographic References

1. Rajkonwar A, Kusre G. Morphological Variations of the Thyroid Gland among the People of Upper Assam Region of Northeast India: A Cadaveric Study. Journal of clinical and diagnostic research : JCDR. 2016;10:AC01-AC3.
2. Sharma M-P, Cetera B. Thyroid disease and surgery. Anaesthesia & Intensive Care Medicine. 2020;21(11):558-71.
3. Viduetsky A, Herrejon CL. Sonographic Evaluation of Thyroid Size: A Review of Important Measurement Parameters. 2019;35(3):206-10.
4. Chaudhary V, Bano S. Thyroid ultrasound. Indian J Endocrinol Metab. 2013;17(2):219-27.
5. Beynon ME, Pinneri K. An Overview of the Thyroid Gland and Thyroid-Related Deaths for the Forensic Pathologist. 2016;6(2):217-36.
6. Al-Azzawi A, Takahashi T. Anatomical variations of the thyroid gland: An experimental cadaveric study. Annals of Medicine and Surgery. 2021;70:102823.
7. Chen Y, Zhou C, Bian Y, Fu F, Zhu Ba, Zhao X, et al. Cadmium exposure promotes thyroid pyroptosis and endocrine dysfunction by inhibiting Nrf2/Keap1 signaling. Ecotoxicology and Environmental Safety. 2023;249:114376.
8. Balasubramanian SP. Anatomy of the thyroid, parathyroid, pituitary and adrenal glands. Surgery (Oxford). 2020;38(12):758-62.
9. Liao B, Liang J, Guo B, Jia X, Lu J, Zhang T, et al. ILSHIP: An interpretable and predictive model for hypothyroidism. Computers in Biology and Medicine. 2023;154:106578.
10. Armstrong M, Asuka E, Fingeret A. Physiology, Thyroid Function. StatPearls. Treasure Island (FL): StatPearls Publishing
Copyright © 2022, StatPearls Publishing LLC.; 2022.
11. Sahin L, Keloglan Müsüroglu S, Selin Cevik O, Cevik K, Orekici Temel G. Hyperthyroidism leads learning and memory impairment possibly via GRIN2B expression alterations. Brain Research. 2023;1802:148209.
12. Solmunde E, Falstie-Jensen AM, Lorenzen EL, Ewertz M, Reinertsen KV, Dekkers OM, et al. Breast cancer, breast cancer-directed radiation therapy and risk of hypothyroidism: A systematic review and meta-analysis. The Breast. 2023.
13. Kobaly K, Kim CS, Mandel SJ. Contemporary Management of Thyroid Nodules. Annual review of medicine. 2022;73:517-28.
14. Nguyen QT, Lee EJ, Huang MG, Park YI, Khullar A, Plodkowski RA. Diagnosis and treatment of patients with thyroid cancer. American health & drug benefits. 2015;8(1):30-40.
15. Norris JJ, Farci F. Follicular Adenoma. StatPearls. Treasure Island (FL): StatPearls Publishing
Copyright © 2023, StatPearls Publishing LLC.; 2023.
16. McHenry CR, Phitayakorn R. Follicular adenoma and carcinoma of the thyroid gland. The oncologist. 2011;16(5):585-93.
17. Soares P, Póvoa AA, Melo M, Vinagre J, Máximo V, Eloy C, et al. Molecular Pathology of Non-familial Follicular Epithelial-Derived Thyroid Cancer in Adults: From RAS/BRAF-like Tumor Designations to Molecular Risk Stratification. Endocrine pathology. 2021;32(1):44-62.
18. Tavares C, Melo M, Cameselle-Teijeiro JM, Soares P, Sobrinho-Simões M. ENDOCRINE TUMOURS: Genetic predictors of thyroid cancer outcome. European journal of endocrinology. 2016;174(4):R117-26.
19. Laha D, Nilubol N, Boufraqech M. New Therapies for Advanced Thyroid Cancer. Frontiers in endocrinology. 2020;11:82.
20. Vasileiadis I, Boutzios G, Karalaki M, Misiakos E, Karatzas T. Papillary thyroid carcinoma of the isthmus: Total thyroidectomy or isthmusectomy? American journal of surgery. 2018;216(1):135-9.
21. Donaldson LB, Yan F, Morgan PF, Kaczmar JM, Fernandes JK, Nguyen SA, et al. Hobnail variant of papillary thyroid carcinoma: a systematic review and meta-analysis. Endocrine. 2021;72(1):27-39.
22. LiVolsi VA. Papillary thyroid carcinoma: an update. Modern Pathology. 2011;24:S1-S9.

23. Haroon Al Rasheed MR, Xu B. Molecular Alterations in Thyroid Carcinoma. Surgical pathology clinics. 2019;12(4):921-30.
24. Wei X, Wang X, Xiong J, Li C, Liao Y, Zhu Y, et al. Risk and Prognostic Factors for BRAF^{V600E} Mutations in Papillary Thyroid Carcinoma. BioMed Research International. 2022;2022:9959649.
25. Melo M, Rocha AGd, Vinagre J, Sobrinho-Simões M, Soares P. Coexistence of TERT Promoter and BRAF Mutations in Papillary Thyroid Carcinoma: Added Value in Patient Prognosis? 2015;33(6):667-8.
26. Abdullah MI, Junit SM, Ng KL, Jayapalan JJ, Karikalan B, Hashim OH. Papillary Thyroid Cancer: Genetic Alterations and Molecular Biomarker Investigations. International journal of medical sciences. 2019;16(3):450-60.
27. Badulescu CI, Piciu D, Apostu D, Badan M, Piciu A. FOLLICULAR THYROID CARCINOMA - CLINICAL AND DIAGNOSTIC FINDINGS IN A 20-YEAR FOLLOW UP STUDY. Acta endocrinologica (Bucharest, Romania : 2005). 2020;16(2):170-7.
28. Filetti S, Durante C, Hartl D, Leboulleux S, Locati LD, Newbold K, et al. Thyroid cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up†. Annals of oncology : official journal of the European Society for Medical Oncology. 2019;30(12):1856-83.
29. Xu B, Ghossein R. Poorly differentiated thyroid carcinoma. Seminars in diagnostic pathology. 2020;37(5):243-7.
30. Volante M, Lam AK, Papotti M, Tallini G. Molecular Pathology of Poorly Differentiated and Anaplastic Thyroid Cancer: What Do Pathologists Need to Know? Endocrine pathology. 2021;32(1):63-76.
31. Dierks C, Seufert J, Aumann K, Ruf J, Klein C, Kiefer S, et al. Combination of Lenvatinib and Pembrolizumab Is an Effective Treatment Option for Anaplastic and Poorly Differentiated Thyroid Carcinoma. Thyroid : official journal of the American Thyroid Association. 2021;31(7):1076-85.
32. Molinaro E, Romei C, Biagini A, Sabini E, Agate L, Mazzeo S, et al. Anaplastic thyroid carcinoma: from clinicopathology to genetics and advanced therapies. Nature reviews Endocrinology. 2017;13(11):644-60.
33. Abe I, Lam AK. Anaplastic thyroid carcinoma: Updates on WHO classification, clinicopathological features and staging. Histology and histopathology. 2021;36(3):239-48.
34. Jayasinghe R, Basnayake O, Jayarajah U, Seneviratne S. Management of medullary carcinoma of the thyroid: a review. The Journal of international medical research. 2022;50(7):3000605221110698.
35. Garcia-Martin G, Sanz-Rodriguez M, Alcover-Sanchez B, Pereira MP, Wandosell F, Cubelos B. R-Ras1 and R-Ras2 Expression in Anatomical Regions and Cell Types of the Central Nervous System. International journal of molecular sciences. 2022;23(2).
36. Clavaín L, Fernández-Pisonero I, Movilla N, Lorenzo-Martín LF, Nieto B, Abad A, et al. Characterization of mutant versions of the R-RAS2/TC21 GTPase found in tumors. Oncogene. 2023;42(5):389-405.
37. Yu C, Lyn N, Li D, Mei S, Liu L, Shang Q. Clinical analysis of Noonan syndrome caused by RRAS2 mutations and literature review. European Journal of Medical Genetics. 2023;66(1):104675.
38. Weber SM, Carroll SL. The Role of R-Ras Proteins in Normal and Pathologic Migration and Morphologic Change. The American journal of pathology. 2021;191(9):1499-510.
39. Hortal AM, Oeste CL, Cifuentes C, Alcoceba M, Fernández-Pisonero I, Clavaín L, et al. Overexpression of wild type RRAS2, without oncogenic mutations, drives chronic lymphocytic leukemia. Molecular Cancer. 2022;21(1):35.
40. Fernández-Pisonero I, Clavaín L, Robles-Valero J, Lorenzo-Martín LF, Caloto R, Nieto B, et al. A hotspot mutation targeting the R-RAS2 GTPase acts as a potent oncogenic driver in a wide spectrum of tumors. Cell reports. 2022;38(11):110522.
41. Waksal JA, Bruedigam C, Komrokji RS, Jamieson CHM, Mascarenhas JO. Telomerase-targeted therapies in myeloid malignancies. Blood Advances. 2023;7(16):4302-14.
42. Chen Y. The structural biology of the shelterin complex. Biological chemistry. 2019;400(4):457-66.
43. Bhari VK, Kumar D, Kumar S, Mishra R. Shelterin complex gene: Prognosis and therapeutic vulnerability in cancer. Biochemistry and biophysics reports. 2021;26:100937.

44. Cong YS, Wright WE, Shay JW. Human telomerase and its regulation. Microbiology and molecular biology reviews : MMBR. 2002;66(3):407–25, table of contents.
45. Hafezi F, Perez Bercoff D. The Solo Play of TERT Promoter Mutations. Cells. 2020;9(3).
46. Vinagre J, Almeida A, Pópulo H, Batista R, Lyra J, Pinto V, et al. Frequency of TERT promoter mutations in human cancers. Nature communications. 2013;4(1):2185.
47. Liu R, Xing M. TERT promoter mutations in thyroid cancer. Endocrine-related cancer. 2016;23(3):R143–55.
48. Henslee G, Williams CL, Liu P, Bertuch AA. Identification and characterization of novel ACD variants: modulation of TPP1 protein level offsets the impact of germline loss-of-function variants on telomere length. Cold Spring Harbor molecular case studies. 2021;7(1).
49. Liu B, He Y, Wang Y, Song H, Zhou ZH, Feigon J. Structure of active human telomerase with telomere shelterin protein TPP1. Nature. 2022;604(7906):578–83.
50. Amir M, Khan P, Queen A, Dohare R, Alajmi MF, Hussain A, et al. Structural Features of Nucleoprotein CST/Shelterin Complex Involved in the Telomere Maintenance and Its Association with Disease Mutations. Cells. 2020;9(2).
51. Bisht K, Smith EM, Tesmer VM, Nandakumar J. Structural and functional consequences of a disease mutation in the telomere protein TPP1. Proceedings of the National Academy of Sciences of the United States of America. 2016;113(46):13021–6.
52. Chun-On P, Hinchie AM, Beale HC, Gil Silva AA, Rush E, Sander C, et al. TPP1 promoter mutations cooperate with TERT promoter mutations to lengthen telomeres in melanoma. Science (New York, NY). 2022;378(6620):664–8.
53. Integrated genomic characterization of papillary thyroid carcinoma. Cell. 2014;159(3):676–90.
54. Landa I, Ibrahimpasic T, Boucai L, Sinha R, Knauf JA, Shah RH, et al. Genomic and transcriptomic hallmarks of poorly differentiated and anaplastic thyroid cancers. The Journal of clinical investigation. 2016;126(3):1052–66.
55. . Available from: https://www.gtexportal.org/home/gene/RRAS2.
56. . Available from: https://www.proteinatlas.org/ENSG00000133818-RRAS2.
57. . Available from: https://hive.biochemistry.gwu.edu/biomuta/proteinview/P62070.
58. . Available from: a POT1-binding domain with a centred position (PBD) and a C-terminal TIN2-binding domain (TBD).
59. . Available from: https://www.ncbi.nlm.nih.gov/gene/65057.
60. . Available from: https://www.uniprot.org/uniprotkb/Q5EE38/entry.
61. . Available from: https://www.gtexportal.org/home/gene/ACD.
62. . Available from: https://www.proteinatlas.org/ENSG00000102977-ACD.
63. . Available from: https://hive.biochemistry.gwu.edu/biomuta/proteinview/Q96AP0.
64. . Available from: https://www.cbioportal.org.
65. Guo Y, Kartawinata M, Li J, Pickett HA, Teo J, Kilo T, et al. Inherited bone marrow failure associated with germline mutation of ACD, the gene encoding telomere protein TPP1. Blood. 2014;124(18):2767–74.
66. Grill S, Bisht K, Tesmer VM, Shami AN, Hammoud SS, Nandakumar J. Two Separation-of-Function Isoforms of Human TPP1 Dictate Telomerase Regulation in Somatic and Germ Cells. Cell reports. 2019;27(12):3511–21.e7.
67. Aoude LG, Pritchard AL, Robles-Espinoza CD, Wadt K, Harland M, Choi J, et al. Nonsense mutations in the shelterin complex genes ACD and TERF2IP in familial melanoma. Journal of the National Cancer Institute. 2015;107(2).
68. Mazzolini R, Gonzàlez N, Garcia-Garijo A, Millanes-Romero A, Peiró S, Smith S, et al. Snail1 transcription factor controls telomere transcription and integrity. Nucleic acids research. 2018;46(1):146–58.