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PhD Thesis in Medicine and Molecular Oncology

ROle of the MITOchondrial fission protein DRP1 as a prognosis and predictive biomarker in the treatment of differentiated thyroid cancer (ROMITO-DRP1)

Papel da proteína de fissão mitocondrial DRP1 como biomarcador prognóstico e predictivo no tratamento do carcinoma diferenciado da tiroide (ROMITO-DRP1)

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ROle of the MITOchondrial fission protein DRP1 as a prognosis and predictive biomarker in the treatment of differentiated thyroid cancer (ROMITO-DRP1)

“Γένοι’ οἷος ἐσσί μαθών”

Πίνδαρος

Píndaro, Ode Pítica II

“Having learned, become who you are”

Nehamas, Alexander: The Art of Living. Socratic Reflections from Plato to Foucault.
Berkeley 1998, p. 128.

“Tornai-vos o que sois, depois de terdes aprendido o que isso é”

Prideaux, Sue: Eu Sou Dinamite! A Vida de Friedrich Nietzsche.
Tradução: Artur Lopes Cardoso. Bertrand Editora
2019, p. 65.

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List of Abbreviations and Acronyms

AD – Alzheimer's disease

AJCC – American Joint Committee on Cancer

Aldh1A1 – aldehyde dehydrogenase 1A1

AKT – Akt serine/threonine kinase

ALR – augmenter of liver regeneration

AMES – Age, Metastasis, Extent of disease, Size

AMP – adenosine monophosphate

AMPK – AMP-activated protein kinase

ATA – American Thyroid Association

ATF1 – activating transcription factor 1

ATM/Chk1 and ATM/Chk2 – ataxia-telangiectasia mutated/checkpoint kinases 1 and 2

ATP – adenosine triphosphate

BAK – bcl-2 antagonist killer protein

BAX – bcl-2-associated X protein

BCL-2 – B-cell CLL/ lymphoma 2 protein

BID – BH3-interacting domain death agonist

BIM – BCL-2-like protein 11

BNIP3 – BLC-2 nineteen-kilodalton interacting protein 3

BRAF / *BRAF* – B-Raf proto-oncogene protein / gene

$BRAF^{Wt}$ – wild-type B-Raf proto-oncogene

BTICs – brain tumor initiating cells

Ca^{2+} – calcium ion

CAMK-Iα – Ca2+/calmodulin-dependent protein kinase

cAMP – cyclic adenosine monophosphate

c-MYC – cellular myelocytomatosis oncogene protein

c-KIT – v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog

CCCP – m-chlorophenylhydrazone

CDDP – cisplatin

cDNA – complementary deoxyribonucleic acid

CH3OH – methanol

COI – cytochrome c oxidase subunit I

CO2 – carbon dioxide

CytB – cytochrome B

cPTC – classic papillary thyroid carcinoma

CRC – colorectal carcinoma

CREB – cAMP responsive element binding protein

dH20 – distillated water

DMEM – Dulbecco's modified Eagle's medium CLPP – caseinolytic protease

DNA – deoxyribonucleic acid

Dnm1 – dynamin 1 protein

Dnml1 – dynamin-1-like gene

DRP1 and DRPs – dynamin-related protein 1 and dynamin-related proteins

DTC – differentiated thyroid carcinoma

DUSP6 – dual specificity phosphatase 6

EBRT – external beam radiotherapy

EC50 – half maximal effective concentration

ERR – estimated risk of recurrence

EGFR-RAS – epidermal growth factor receptor (EGFR)- retrovirus associated sequence oncogene (RAS) signaling pathway

EOC – epithelial ovarian cancer

EORTC - The European Organization for the Research and Treatment of Cancer

ER – endoplasmic reticulum

ERK1 and ERK2 – extracellular signal–regulated kinase 1 and 2

ETC – electron transport chain

^{18}FDG –18F-fluorodeoxyglucose

FGFR – Fibroblast growth factor receptor

FIS1 – fission 1 protein

FNAC – fine needle aspiration cytology

FOXM1 – forkhead box protein M1

FOXO3 – forkhead box O3 protein

FTC – follicular thyroid carcinoma

FVPTC – papillary thyroid carcinoma

GDAP1 – ganglioside-induced differentiation-associated protein 1

GSK3β – Glycogen synthase kinase 3 beta

GTP – guanosine triphosphate

GTPase – guanosine triphosphatase

IARC – international agency for research on cancer

l – long

LB – loading buffer

HBME1 – Hector Battifora mesothelial-1

HepCC – hepatocellular cell carcinoma

HCC – Hürthle cell carcinoma

HEK – human embryonic kidney

hFIS1– human fission 1 protein

HIF-α – hypoxia-inducible factor 1α

H2O2 – hydrogen peroxide

hTert – human telomerase reverse transcriptase

IMM – inner mitochondrial membrane

INF2 – inverted formin-2 protein

iPSCs – induced pluripotent stem cells

iRNA – intereference ribonucleic acid

IUAC – International Union Against Cancer

KLF4 – kruppel-like factor 4

LDH-A – lactate dehydrogenase A

MACIS – Metastases, Age, Completeness of resection, Invasion, Size

MAPL – mitochondrial-anchored protein ligase

MAPK – mitogen-activated protein kinase

MARCH5 – membrane associated ring-CH-type finger 5

mCi – milicurie

Mdivi-1 – mitochondrial division 1 inhibitor

MEFs – mouse embryonic fibroblasts

MEK – mitogen-activated protein kinase (MAPK)/ERK kinase

NFAT – nuclear factor of activated T-cells

Mgm 1 – mitochondrial genome maintenance

MIEF1– mitochondrial elongation factor 1

MFF – mitochondrial fission factor

MFN 1/2 – mitofusin 1/2

MiD49 and MiD51 – mitochondrial dynamics protein of 49 kDa and 51kDa

MTP18 – mitochondrial protein, 18 kDa

Myc – MYC proto-oncogene

ND1 – ubiquinone oxireductase chain 1

NIS – sodium iodide symporter

mRNA – messenger ribonucleic acid

mtDNA – mitochondrial deoxyribonucleic acid

NAD+ – nicotinamide adenine dinucleotide

NCCN – The National Comprehensive Cancer Network

NF-kB – nuclear factor kappa B

NO – nitric oxide

NRF1 – nuclear respiratory factor 1

OCT4 – octamer-binding factor 4

OMM – outer mitochondrial membrane

OPA1 – optic atrophy 1

OS – overall survival

ovPTC – oncocytic variants of papillary thyroid carcinoma

OXPHOS – oxidative phosphorylation

p53 – tumor protein p53

PAGE – polyacrylamide gel electrophoresis

PARK2 – Parkinson protein 2 E3 ubiquitin protein ligase gene

PARKIN – Parkinson protein 2, E3 ubiquitin protein ligase

PAX8 – paired box gene 8

PB – presto blue

PBS – phosphate buffered saline

PPARɣ– peroxisome proliferator-activated receptor gamma

PDGF-R-β – platelet-derived growth factor receptor beta

PDK1 – pyruvate dehydrogenase kinase 1

PDTC – poorly differentiated thyroid carcinoma

p-ERK – phosphor-ERK

PET – positron emission tomography

PGC1-α – peroxisome proliferator-activated receptor γ coactivator 1-alpha

PI – propidium iodine

PI3K – phosphoinositide 3-kinase

PIK3CA – phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha

PKA – protein kinase A

PKC – protein kinase C

PTC – papillary thyroid carcinoma

PTEN – phosphatidylinositol-3,4,5-triphosphate 3-phosphatase

RAF – radiofrequency ablation

RAI – radioiodine

RALBP1 – recombinant protein of human ralA binding protein 1

RAS – Rat sarcoma virus

RET – ret proto-oncogene

rhTSH – recombinant human thyroid-stimulating hormone

RIPA – reagent-based cell lysis using radioimmunoprecipitation

ROCK – rho-associated coiled-coil kinase

ROS – reactive oxygen species

RPMI – Roswell Park Memorial Institute

RQ-PCR – real-time quantitative polymerase chain reaction

s – short

Ser – serine residues

SD – standard deviation

SDS – sodium dodecyl sulfate

SIRT1 – sirtuin 1

siRNA – small interfering ribonucleic acid

shRNA – short hairpin ribonucleic acid

SOX2 – sex determining region Y-box 2

S616-p-DRP1– Serine 616-phosphorylated DRP1

SRB – sulforhodamine

TERT – telomerase reverse transcriptase

TFAM – mammalian mitochondrial transcription factor

TC – thyroid carcinoma

TCGA – The Cancer Genome Atlas

TDS – thyroid differentiation score

Tg – thyroglobulin

TKI – tyrosine kinase inhibitor

TNM – Tumor, Node, Metastasis

TP53 – tumor protein p53 gene

TPO – thyroid peroxidase

TSH – thyroid-stimulating hormone

TSHr – thyroid-stimulating hormone receptor

TTF-1 – thyroid transcription factor 1

UCP2 – mitochondrial uncoupling protein 2

US – ultrasound

UTC – undifferentiated thyroid carcinoma

VEGFR-1-3, – vascular endothelial growth factor-1, -2, -3

WBS – whole-body scan

WDTC – well-differentiated thyroid carcinoma

Abstract

Risk stratification in thyroid cancer (TC) is an evolving field with an unmet need for prognostic factors which can predict poor outcomes. Dynamin-related protein 1 (DRP1), a member of the dynamin family of guanosine triphosphatases (GTPases), is the key component of mitochondrial fission machinery. DRP1 is associated with different cell processes, such as apoptosis, mitochondrial biogenesis, mitophagy, metabolism, cell proliferation, differentiation, and transformation. DRP1 and its active form phosphorylated at Serine 616 (S616-p-DRP1) have been associated with the development of distinct human cancers through their action in different biological processes, including mitochondrial energetics and cell metabolism, cell proliferation, stem cell maintenance, invasion, and promotion of metastases.

We aimed to assess the expression of DRP1, and later S616-p-DRP1, by immunohistochemistry in a large series of tumors from patients with follicular cell-derived thyroid carcinoma (FCDTC) and explore if they could be a prognostic candidate in TC. We have also explored the effects of DRP1 pharmacological inhibition alone or in combination with mitogen-activated protein kinase (MAPK) inhibition in various TC cell line models. Although there was no significant correlation between DRP1 and S616-p-DRP1 expression, both were associated with locally invasive characteristics of the tumor, with S616-p-DRP1 expression showing a stronger and significant association with tumor locally invasive behavior and lymph node metastases. Unexpectedly, S616-p-DRP1 expression was negatively associated with the oncocytic phenotype. The *in vitro* pharmacological modulation of DRP1 seems to be deleterious in the oncocytic tumors at the differentiation level with a decrease in the expression of differentiation markers, such as sodium-iodine symporter (NIS) and thyroid-stimulating hormone receptor (TSHr), while the BRAF inhibitor dabrafenib increases these differentiation markers in oncocytic tumors. The modulation of mitochondrial dynamics targeting DRP1 may be advantageous when combined with a MAPK inhibitor, such as

dabrafenib, which can revert radioiodine resistance, a strategy that may not be effective in Hürthle cell carcinoma (HCC).

In conclusion, S616-p-DRP1 is a better prognostic candidate than DRP1 to identify tumors with locally invasive behavior, and as a putative differentiator for those with systemic involvement. We propose that S616-p-DRP1 expression is validated in prospective studies as a candidate biomarker for the stratification of pre- and post-operative risk assessment in patients with differentiated thyroid carcinoma (DTC). We also suggest that studies addressing radioiodine resistance and cell death in HCC focus on the modulation of mitochondrial dynamics and metabolism.

Resumo

A estratificação de risco no carcinoma da tiróide representa uma área em evolução, onde existe uma necessidade não satisfeita relativamente a factores de prognóstico que possam predizer resultados clínicos desfavoráveis. A proteína relacionada com a dinamina 1 (DRP1), membro da família das dinamina guanosina trifosfatases (GTPases), é uma componente chave da maquinaria de fissão mitocondrial. A DRP1 encontra-se associada a diferentes processos celulares, como a apoptose, biogénese mitocondrial, mitofagia, metabolismo, proliferação celular, diferenciação e transformação maligna. A DRP1 e a sua forma activa fosforilada no resíduo 616 de serina (S616-p-DRP1) foram associadas ao desenvolvimento de diferentes tumores malignos humanos, através do seu papel em vários processos biológicos, incluindo alterações na energia e metabolismo celular, proliferação celular, manutenção do estado estaminal, invasão e promoção de metástases.

Propusemo-nos a avaliar a expressão de DRP1 e, posteriormente, de S616-p-DRP1, por imunohistoquímica numa grande série de tumores de doentes com carcinomas derivados das células foliculares da tiroide, bem como a explorar se aquelas proteínas poderiam ser consideradas como candidatos de valor prognóstico no carcinoma da tiroide. Explorámos igualmente os efeitos da inibição farmacológica, isoladamente ou em combinação com um inibidor da via da MAPK, em diferentes modelos de linhas celulares de carcinoma da tiroide.

Embora não se tenha verificado uma correlação significativa entre a expressão da DRP1 e S616-p-DRP1, ambas as proteínas se associaram a características localmente invasivas dos tumores, tendo a expressão da S616-p-DRP1 apresentado uma associação maior, e significativa, com um comportamento localmente invasivo do tumor e metastização ganglionar. Surpreendentemente, a expressão de S616-p-DRP1 associou-se de forma negativa ao fenótipo oncocítico. A modulação farmacológica da DRP1 *in vitro* parece ser deletéria nos tumores oncocíticos ao nível da diferenciação, com uma redução da expressão de marcadores como o co-transportador sódio-iodeto (NIS) e o receptor da hormona

estimuladora da tiróide (TSHr), por oposição ao inibidor do BRAF dabrafenib, que aumenta a sua expressão nestes tumores. A modulação da dinâmica mitocondrial tendo como alvo a DRP1 pode apresentar vantagens quando associada a um inibidor da via da MAPK que reverta a resistência ao iodo, como é o caso do dabrafenib, uma estratégia que pode, porém, não ser eficaz no HCC.

Em conclusão, a S616-p-DRP1 apresenta-se como um melhor candidato de valor prognóstico para diferenciar tumores com um comportamento localmente invasivo, e como um putativo diferenciador de tumores com envolvimento sistémico, por comparação com a DRP1. Propomos que a expressão de S616-p-DRP1 seja validada em estudos prospectivos como biomarcador para a estratificação de risco no contexto pré- e pós-operatório em doentes com carcinoma diferenciado da tiroide (DTC). Sugerimos ainda que estudos que explorem o problema da resistência ao iodo e da morte celular no carcinoma de células de Hürthle (CCH) incluam a modulação da dinâmica mitocondrial e do metabolismo.

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Statement

According to Art.º 8º do Decreto-Lei n.º 388/70, the following body of work are part of the Thesis:

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Poster presentation (national)

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> sob a luz do farol iluminado que naquelas noites estivais acendemos na Quinta de Marim, onde cheiros de pinheiro manso se enamoram do sabor das salinas, no silêncio gritado do apitar longínquo do comboio quebrado apenas pelas ondas que batem no Moinho

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1. Introduction

1.1 State of the art in the diagnosis and clinical management of differentiated thyroid cancer

Epidemiology

Thyroid carcinoma (TC) is the most common endocrine malignancy, with a worldwide 5-year prevalence by 2018 of approximately 4.6% of all malignancies (https://gco.iarc.fr/today/online-analysis-pie, acceded 12 December, 2019). In Europe, the age standardized rate (ASR) of new cases of TC is estimated to range from 4.4 to 13.9 per 100,000 person-years in men and women, respectively (1), while in the USA the numbers range from 8.0 to 23.2 per 100,000 person-years, respectively (2). The reported increasing incidence in the last decades is explained by the better detection of small papillary carcinomas as a result of improved diagnostic methods, in particularly by the generalized use of imaging techniques, fine needle aspiration (FNA) and medical surveillance. These have allowed the identification of subclinical papillary thyroid carcinomas (PTCs) (2-4). Nevertheless, the incidence rates seem to have recently stabilized, as reported in the United States of America (USA), possibly due to more restrictive recommendations for thyroid biopsy and the reclassification of the previously named encapsulated non-invasive follicular PTC variants as non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) (5).

TC affects women more frequently than men, at a ratio of 3:1 (1, 2). One study in the USA has predicted that PTC would become the third most common cancer in women in 2019 (6). In fact, it was already the third most common cancer in women in Portugal back in 2010, as reported by the National Cancer Registry (RON) (https://www.dgs.pt/, accessed 22 February, 2020). Mortality is very low, varying between 0.5 and 0.6 in both genders, in the US and Europe, respectively, with less variation between regions and time than what is observed for incidence rates (1, 2, 7). Still, mortality rates are lower in young women as compared to

men (2, 8, 9). Differentiated thyroid carcinomas (DTC) have a 10-year survival which can exceed 90 to 95% (10).

Exposure to ionizing radiation is the only established environmental risk factor (11). It seems that the risk of radiation-induced TC is higher in women, patients with a previous family history of TC and the Jewish population (12). Other factors, such as TSH levels, autoimmune thyroiditis, iodine intake or deficit, obesity, and diet and environmental pollutants have been described as having a potential role in the risk of TC, but a clear relationship has not yet been defined (11, 13).

Diagnosis: pathology and molecular aspects

TC is classified according to the cell type it derives from, degree of differentiation and cytoarchitecture. Resected differentiated thyroid carcinomas (DTCs) are histologically classified according to the World Health Organization (WHO) criteria (Table 1) (14). Follicular-cell derived tumors comprise well differentiated (WDTC), poorly differentiated (PDTC) and undifferentiated thyroid carcinoma (UTC). The well differentiated group includes follicular thyroid carcinoma (FTC) and papillary thyroid carcinoma (PTC), the latter having two main variants: classic (cPTC) and follicular variant (FVPTC). The minority of carcinomas that derive from parafollicular C cells are called medullary thyroid carcinomas. According to the previous 3th edition of "WHO (World Health Organization) Classification of Tumors of Endocrine Organs", tumors composed by more than 75% of oncocytes are classified as variants of papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC) (15). However, under the most recent 4th edition WHO classification, oncocytic thyroid neoplasms with follicular architecture but no typical nuclei of papillary carcinoma are now included in a separate group - the so called Hürthle cell neoplasms (16). Oncocytes terminology is used to describe the appearance of a thyrocyte as a result of a significant increase in the amount of abnormally swelled mitochondria due to the mitochondrial dysfunction (17, 18).

The diagnosis of TC includes the use of thyroid ultrasound (US) followed by fine needle aspiration cytology (FNAC) (3). FNA diagnosis can be supported by the assessment of

markers such as HBME1 or galectin-3 and genetic alterations associated with malignancy, such as B-Raf proto-oncogene (*BRAF*) mutations or ret proto-oncogene (*RET*) fusions. Some gene panels have been reported to be useful for the diagnosis of identifying malignancy when cytology results are indeterminate, but these are seldom used in clinical practice (19).-The use of immunohistochemical markers in cytological samples to differentiate between various histologic subtypes has been used with poor results (3, 20-23). Some reports have postulated the potential use of molecular testing for diagnosis since 97% of nodules bearing mutations in genes such as *BRAF*, *RAS*, *RET/PTC* and paired box gene (*PAX8)*/Peroxisome Proliferator-Activated Receptor γ (*PPARγ*) had malignant diagnosis (24). A study by The Cancer Genome Atlas (TCGA) Research Network confirmed the existence of two main genetic types of PTC, which correspond to the cPTC and some of its variants, and the follicular variants of PTC (FVPTC) (25). The former display $BRAF^{V600}$ mutations and *RET/PTC* rearrangements, and the latter are associated with *RAS* mutations and *PAX8/PPARɣ* rearrangements (26). $BRAF^{V600E}$ is the most prevalent mutation and is characteristic of classic PTC, or its variants, where it is present in 36- 83% of the cases (27). There have been several lines of evidence associating this mutation with clinico-pathological indicators of poorer prognosis, whilst others have not supported such link (28-35). An association has been described between this mutation and the loss of radioactive iodine avidity in recurrent PTC (32, 36-38). The clinical use of an investigational MEK inhibitor, selumetinib, as well of the *BRAF* inhibitor dabrafenib, restored radioiodine (RAI) incorporation, further building on the concept that *BRAF* mutation is associated with a decrease of thyroid specific genes or “Iodine handling genes (26, 39, 40). Nevertheless, the role of routine *BRAF* mutations assessment in PTCs has not yet been clarified *(3)*. *RAS* mutations are more prevalent in less differentiated tumors where they seem to be more relevant as prognostic indicator, specifically associated with distant metastases and lower survival. However, data is still missing to define its role as a prognostic factor (41-43). Its prevalence is higher in FTC (36%), PDTC (55%) and UTC (52%), and lower in PTC (10%) (44-46). The prognostic value of *RET/PTC* and *PAX8/PPAR* rearrangements has not yet been fully clarified (26, 27, 47, 48).

Approximately, two thirds of TC display telomerase activation, and there have been recent reports of mutations in the promoter region of telomerase (TERT) gene in a large thyroid tumor cohort of samples, with an increasing relative prevalence from well to poorly differentiated and undifferentiated carcinomas (49, 50). *TERT* promoter mutations seem to be more frequent in *BRAF* mutated PTC tumors. Several retrospective studies have pointed out to the association between *TERT* promoter mutations and clinico-pathological features of poorer prognosis, including distant metastases (50-53). Mutations of *TP53* have been widely described in PDTC or UTC, where rates vary between 26% to 60% respectively. However, this mutation has also been described recently in DTC, with rates between 0.7% and 11.1% in PTC and FTC, respectively (25, 54). Histologic characteristics of aggressiveness support the fact that *TP53* inactivation plays a role in the progression of differentiated to undifferentiated tumors (26). The lethal forms of non-ATC are normally PTC variants with the typical more common mutations already described, with additional gene alterations such as TERT promoter, TP53, and/or genes of PI3K/AKT/mTOR pathway [20].

There are some challenges in the cytology diagnosis of TC. FTC and the recently categorized encapsulated non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) are frequently classified as indeterminate by cytology (14, 22, 23, 55). The latter are classified as such based on low-grade behavior over long-term follow-up (3). Indeed, they were associated with no reports of cancer-related deaths and an estimated risk of recurrence (ERR) of <1% (3). Hürthle cell carcinoma (HCC) are rarely diagnosed by FNAC, as the diagnostic criteria require demonstration of vascular or capsular invasion (56-58). Similarly, diagnosis of PDTC based on FNAC is also challenging unless mitotic activity and/or necrosis are observed.

The most used TC staging classification is the one of the American Joint Committee on Cancer (AJCC)/International Union Against Cancer (IUAC) Tumor, Node, Metastasis (TNM) staging system, based on the extent of tumor and age, which was updated in 2016 (10). Although all staging systems are able to predict high or low risk of cancer mortality, they fail to predict the risk of recurrence (21). The American Thyroid Association (ATA) have

published a risk assessment which estimates the risk of recurrence, assigning the risk of persistent or recurrent disease as low (≤5% ERR), medium (6-20% ERR) or high (>20% ERR) based on data available right after the treatment of the primary tumor, including pathology staging, histology and clinical features, such as the result of the post-ablative whole-body scan (WBS) and serum thyroglobulin (Tg) assessment (55). This risk assessment should then be revised during follow-up of the patient to include the disease evolution and response to treatment, providing a dynamic risk stratification (23).

Table 1 Follicular cell-derived TC WHO classification and molecular characterization

Well-differentiated thyroid carcinoma (WDTC)
Papillary thyroid carcinoma (PTC) and variants
$BRAF^{V600E}$, RET/PTC, RAS, TRK
Follicular patterned carcinomas
Follicular cell carcinoma (FTC)
Follicular variant of PTC (FVPTC)
RAS, PAX8/PPARγ, PI3K, PTEN, BRAF
Poorly differentiated thyroid carcinoma (PDTC)
RAS, TP53, Beta-cantenin , BRAF, PI3K, AKT
Anaplastic thyroid carcinoma (ATC)
TP53, Beta-cantenin, RAS, BRAF, PI3K, AKT, PTEN

Prognosis

TC presents an overall good prognosis, with a 2018 age-standardized rate (ASR) incidence in Europe of 9.3 per 100,00 person-years and an ASR mortality of only 0.6 per 100,000 person-years (1). In the USA, TC represents 3.1% of new malignant tumors, but accounts for only 0.4% of deaths due to cancer (2). Worldwide, it represents the 9th cause of

new malignancies, but it ranks 24th as cause of death due to cancer (https://gco.iarc.fr/today/data/factsheets/cancers/32-Thyroid-fact-sheet.pdf, accessed 12 December 2019). The overall prognosis of patients with DTCs is usually very good, with a 10-year survival rate higher than 90% (59). However, it can go down to 10% in patients who develop metastases and have radioiodine-refractory disease (60, 61).

The mortality rates for PTC and FTC are very similar when we compare identical ages and tumor stage. If tumors are small, confined to thyroid and minimally invasive, prognosis is considered to be very good. The same patients may have a much worse prognosis if their disease is highly invasive or if they have distant metastases (62, 63).

Although many factors contribute to the prognosis of DTC, patient's age at the moment of treatment is a key prognostic factor for TC mortality, with an increase in risk of death after 40 years of age, and thereafter with each decade (20). In fact, most staging and prognostic scoring methods use age higher than 40 years as a key aspect to assess mortality risk in DTC (64-68).These scoring approaches differentiate patients with low versus high risk of TC-related mortality, and they include EORTC, TNM 7th edition, AMES and MACIS (64-68). It is known that TC is more aggressive in men (20, 69, 70). Recurrence is highest in patients who are less than 20 years and older than 60 (62, 64, 70-72). However, these scoring systems are far from being perfect as they still fail to identify the so-called low risk patients who will eventually die from TC (73).

Stage of the disease has also prognosis implications, and most physicians who treat this disease recognize age, tumor stage and histological characteristics as critical in determining the therapy and follow-up approach (62, 73-75). The AJCC TNM staging is still the most used system to define TC patients' prognosis (76). It assigns patients who are less than 55 years to stage I and stage II, without or with distant metastases, respectively. Patients 55 years of age or older who have tumors ≤ 4 cm and with no lymph node involvement are also stage I, and those with tumors > 4 confined to thyroid (T3a), and those that have gross extrathyroidal invasion of strap muscle only (T3b), independently from lymph node involvement, and with no distant metastases, are stage II. Patients who are 55 years of age

or older are categorized in stage III if the tumor invades subcutaneous tissue, larynx, trachea, esophagus or recurrent laryngeal nerve (T4a). Stage IV is assigned to patients who are 55 years old, have gross invasion of the prevertebral fascia or tumor invading major vessels (T4b) or those who have distant metastases, independently from tumor size or lymph node involvement (76). Distant metastases are the major reason of death from DTC (77, 78), and half of the cases are present at the moment of diagnosis (72).

According to the 2019 NCCN guidelines, the divergence between cancer-related mortality and frequency of disease recurrence explains some of the disagreements on the best treatment approach for patients with DTC (20). This has also triggered the need to define staging systems which are able to stratify risk of recurrence (79-82).

Regarding tumor histology, it has been documented that PTC with tumor capsule have a better prognosis (83). It is also established that anaplastic tumor transformation, tall-cell papillary variants, as well as columnar papillary variants of PTC, cell diffuse sclerosing variants (83-85) and multinodular (diffuse) form of FVPTC have a worse prognosis (14). A worse prognosis is also usually linked with the diagnosis of FTC with extensive vascular invasion (16, 23). As mentioned previously, NIFTP presents an indolent behaviour and a low risk of an unexpected poor outcome (86-89). As for HCC, it has been defended that this previously assigned variant of FTC can evolve as an aggressive tumor, particularly because of its higher prevalence of distant metastases as compared to FTC (90, 91) and the fact that these tumors seem to be less sensitive to RAI therapy (90). We have recently done a review of the histological, molecular and clinical aspects of the so-called oncocytic tumors, a group which includes HCC (92). A thorough revision of these pathology aspects goes beyond the scope of this dissertation. However, given the observations from our IHC and cell line work in the oncocytic tumors, we chose to present a detailed review of the pathology, molecular and clinical aspects of oncocytic neoplasms (please refer to the full publication in Appendix I).

Treatment

The initial treatment of DTC is surgery, usually total or near-total thyroidectomy, always preceded by exploration of the neck by ultrasound (US) to assess the status of lymph node chains, with or without prophylactic neck dissection, depending on the risk level (3, 20). The use of lobectomy may be defendable in low-risk cases, since it does not seem to be associated with a lower overall survival (OS), and given the non-negatable risk of temporary or permanent hypoparathyroidism and recurrent laryngeal nerve injury (3, 93). The only condition in which active surveillance through US of the thyroid and neck lymph nodes is admitted is in cases of unifocal papillary microcarcinomas, without extracapsular extension nor lymph node metastases (3, 94) . Surgery may be followed by the administration of RAI to ablate any remnant thyroid tissue – which helps in the future patient follow-up -, eliminate potential microscopic residual tumor, thereby acting as an adjuvant treatment, or treating known persistent or recurrent disease (3, 20, 23). RAI is administered after thyroid-stimulating hormone (TSH) stimulation either by levothyroxine withdrawal or by administration of recombinant human TSH (rhTSH) (3, 20, 23). RAI ablation with high activities (≥100 mCi, 3.7 GBq) is indicated in high-risk patients, (7-10, 42), whereas lower activities are used in most intermediate- and low-risk cases (7, 44). Ultimately the decision must be individualized, with RAI doses and TSH stimulation scheme being based on surgical and clinic-pathological considerations (23, 95, 96). Post-surgery thyroid hormone therapy should be initiated to replace thyroid hormone and to suppress the potential growth stimulus of TSH on tumor cells.

Treatment of recurrent loco-regional disease is based on the combination of surgery and RAI therapy, supplemented by external beam radiotherapy (EBRT) if surgery is incomplete or there is lack of RAI uptake. Radiofrequency ablation (RAI) may also be an option for some metastatic lesions, depending on their number, dimension and localization (3, 20, 23).

Systemic therapy can be considered for tumors that are not surgically resectable, responsive RAI, or amenable to RAI, EBRT or other local therapies, and who have clinically significant disease progression over the last 6 to 12 months (3, 20, 23). Indeed, despite an

overall good prognosis, 10–15 % of the DTCs turn refractory to radioactive iodine therapy (69). Up to approximately 20% of patients develop distant metastases, and most of these become refractory to RAI (61, 97). Tyrosine kinase inhibitor (TKIs) that have been tested against DTC in clinical trials include sorafenib, lenvatinib, axitinib, cabozantinib, motesanib, nindetanib, pazopanib, sunitinib vandetanib, as well as the *BRAF*^{V600E} mutation inhibitors vemurafenib and dabrafenib (98-110). Lenvatinib and sorafenib are currently the only agents approved by the European Medicines Agency (EMA) for the treatment of patients with progressive, locally advanced or metastatic RAI-refractory DTC (3, 20, 23). In the randomized phase 3 trial DECISION, sorafenib demonstrated a significant prolongation in median progression-free survival (PFS) of 10.8 months versus 5.8 months with placebo (HR, 0.59; 95% CI, 0.45-0.76; *P* <0.001) (98). However, it did not show any improvement in OS in comparison with placebo (98). For the 12% of patients who had a partial objective response, median duration of response was 10.2 months (95% CI 7.4-16.6) (98). The results were more striking for lenvatinib in its phase 3 trial SELECT, where it showed a median PFS of 18.3 months compared to 3.6 months in the placebo group (HR, 0.21; 99% CI, 0.14 to 0.31; *P*<0.001)(99). However, no OS benefit has been demonstrated (99). Interestingly, 64.8% of patients presented an objective response, with a medium time to response of 2 months (95% CI 1.9-3.5), and in a sub-group analysis lenvatinib showed a significantly improved OS in patients older than 65 years (HR 0.53, 5% CI 0.31-0.91, *P*=0.020) (99, 111). None of these targeted therapies has been documented to be more effective in specific molecular sub-type of tumors, and their multiple targets (VEGFR-1-3, FGFR-1-4, RET, c-KIT and PDGF-R-α for lenvatinib, and VEGFR-1, VEGFR-2, and VEGFR-3, RET, RAF, and PDGF-R-β for sorafenib) make it hard to establish such a correlation. Recently, the United States Food and Drug Administration (FDA) approved two small *BRAF*-specific inhibitors: vemurafenib, for *BRAF*^{V600E}-positive advanced RAI-refractory TC, and dabrafenib in combination with trametinib, a MEK inhibitor, for *BRAF*^{V600E}-positive locally advanced or metastatic ATC. However, these have not yet been approved by EMA.

Background for this project

It has been argued that the prognosis of TC depends more on the interplay between clinical and biological factors, including age, size, gender, histopathological features, and genetic factors, than from genetic factors alone. Both the AJCC/IUAC staging system, which combines age and TNM staging to assess the risk of death due to TC, and clinico-pathologic features are accepted as prognostic indicators in TC. Less consensus exists about the role of genetic or molecular markers as individual prognosis measure. Amongst these, mutations in the telomerase reverse transcriptase (TERT) promoter and in TP53 have been retrospectively associated with a worse clinical outcome, but still require a prospective validation. B-Raf proto-oncogene (BRAF) and rat sarcoma viral oncogene (RAS) may also have a prognosis value under some circumstances, not yet fully clarified.

Recently, our group reported an overall increase in the levels of “mitochondria-shaping” proteins in TC, suggesting a role for abnormal mitochondrial biogenesis and dynamics in thyroid cell transformation (112). From those, dynamin-related protein 1 (DRP1) — the major player in mitochondrial fission — was the most highly expressed in TC, and it was shown to be associated with hallmarks of cancer particularly within the oncocytic malignant tumors, including cell invasion and migration (112). We have proposed to explore the field of prognosis and treatment, following this work. The hypothesis that DRP1 could have clinical implications in the management of DTC, including in prognosis and treatment, was further supported by various reports published in different tumor models, which we will review in the next chapter.

1.2 Dynamin-related protein 1 at the crossroads of cancer

This chapter appears as an article with the same title published in Genes (Lima, Ana Rita *et al*. "Dynamin-Related Protein 1 at the Crossroads of Cancer." *Genes* vol. 9,2 115. 21 Feb. 2018, doi:10.3390/genes9020115).

Introduction

Mitochondrial dynamics is known to have an important role in the so-called age-related diseases, including obesity and type 2 diabetes, Parkinson's disease, Alzheimer's disease (AD), and cancer. Despite this, research on cancer and mitochondrial dynamics has only recently started to be unveiled [1–4].

Mitochondria are organelles involved in many key cellular functions, such as adenosine triphosphate (ATP) production, cell anabolic and catabolic functions, calcium signaling, cell division and differentiation, and cell death [5–7]. Mitochondria respond to physiologic or stress stimuli by adapting their structure and function, which are intimately connected [8]. In recent years, much has been explored on the key molecules and processes that intervene on, or drive, some of these structural and functional changes. Perhaps the most important of such structural changes is the phenomena of mitochondrial fission and fusion, which occur in normal cells, as well as in cells under dysregulation, such as cancer cells, as reviewed by Chen and Chan, and Westermann [9,10]. Mitochondrial fission secures an adequate number of mitochondria to support growing and dividing cells [8,9]. Mitochondrial fission also generates new organelles and represents a quality control mechanism by eliminating damaged mitochondria through selective autophagy, also called mitophagy [9,11]. Mitochondria fusion, on the other hand, is required for maximal ATP production when mitochondria

need to rely on oxidative phosphorylation, or when they have to react to stress stimuli, in which case they appear as elongated healthy organelles that complement the dysfunctional

mitochondria [12–14]. Fusion also allows the exchange of proteins, metabolites and mitochondrial DNA (mtDNA) within the mitochondrial network, avoiding the accumulation of damaged contents in mitochondria [12,15]. Interestingly, Kowald and Kirkwood have proposed mitochondrial fusion as being a permissive mechanism to clonal expansion of mitochondrial deletion mutants, rather than a rescue mechanism for damaged mitochondria [16,17].

Dynamin-related protein 1 (DRP1), a member of the dynamin family of guanosine triphosphatases (GTPases), is the key component of the mitochondrial fission machinery [18]. Dynamin-related protein 1 has been linked to the development of different malignant tumors, including skin, brain, breast, lung, thyroid and endometrial cancer. However, the underlying mechanism(s) for this association is still being explored [19–24]. Dynamin-related protein 1 had roles in changing cellular metabolism in melanoma, contributing to stemness in glioblastoma, involvement with lymph node metastases in breast cancer, sustaining cell cycle and proliferation in lung cancer, and associations with the oncocytic phenotype in thyroid cancer [19–23]. Besides its impact on metabolic regulation, DRP1 has also been associated with a broad range of cell processes: apoptosis, mitochondrial biogenesis and mitophagy, cell proliferation, and differentiation and transformation [19,25–29].

Herein, we review the published knowledge on the role of DRP1 in cancer, exploring its interactions with different biological processes, particularly in the tumorigenesis context. Given the broad range of cellular processes where DRP1 is involved, and its interactions with key known hallmarks of cancer, we will start by reviewing DRP1 role in mitochondria fission and its regulation. Following this, we will provide an overview of DRP1 interplay with biological processes known to be altered in cancer which are important for tumor progression, such as cell death, metabolic programming, and the cell cycle (Table 1). We will then discuss dysregulation of these processes in different tumor models centered on DRP1 alterations, particularly the role of this protein in the invasion and metastization processes, relevant for the generalization stages of tumorigenesis. We will finish with a summary of future perspectives and potential clinical implications of targeting DRP1.

Table 1. Summary of Dynamin-related protein 1 (DRP1) interplay with key cellular processes.

Cell Process	Effects
Cell Death	• DRP1 associates with bcl-2-associated X protein (BAX) at mitochondrial fission sites, promoting permeabilization of the outer mitochondrial membrane (OMM) and cytochrome *c* release [64] • DRP1 drives balance between fission-fusion impacting mitochondrial Ca^{2+} responses in apoptotic signaling [65] • DRP1 inhibition inhibits BAX-BAK dependent cytochrome *c* release [66] • DRP1 knockdown reduces caspase-3 activation and apoptosis [67] • DRP1 inhibition is associated with increase in apoptosis [22]
Metabolic Reprogramming	• DRP1 upregulation associates with less metabolically active mitochondria and increased mitochondrial biogenesis [68] • DRP1 inhibition associates with increased mitochondria oxidative capacity [68]
	Response to hypoxic conditions: • DRP1 expression increased [69] • DRP1 expression decreased after inhibition of HIF-1α [69] • DRP1 inhibition affects HIF1-α expression [69]
	Response to starvation: • Decrease in mitochondrial fraction and activation of $DRP1^{S616}$ through PKA activation [53,70] • Elongation of mitochondria [70] • Shift from glycolysis to oxidative phosphorylation (OXPHOS) [70] • Activation of LDH-A and PDK1 HIF-1α target genes [70] • OXPHOS/glycolysis interchange through HIF-1α /c-MYC pathway [71]
Cell Cycle	• DRP1 functionally or molecularly linked to Cyclin B, E and D [19,29,54,55,72,73] • DRP1 correlates with cell-cycle genes in various cancer types [74] • Mitochondrial morphology is associated with cell cycle control at the G1–S boundary [29,54] • DRP1 inhibition is associated with decrease of cell viability and mitotic program [29,54] • DRP1 knockdown reduces proliferation and percentage of cells in sub-G_0/G_1 cell cycle phase [67] • DRP1 downregulation associates with activation of DNA damage signaling pathways and ATM kinase-dependent G_2/M cell cycle checkpoint, genomic instability and aneuploidy [28] • DRP1 inhibition significantly decreases tumor size [22]

BAX: Bcl-2-associated X protein; BAK: Bcl-2-associated death promoter protein; HIF1-α: hypoxia-inducible factor 1; PKA: protein kinase A; LDH-A: lactate dehydrogenase A; PDK1: pyruvate dehydrogenase kinase 1; c-MYC: myelocytomatosis oncogene protein; ATM: ataxia telangiectasia mutated protein.

Regulation of Dynamin-Related Protein 1 and Its Central Role in Mitochondrial Fission

Mitochondrial fusion and fission proteins, first identified in flies and yeast, are key players in mitochondrial biogenesis [30]. There are three highly conserved dynamin-related GTPases

(DRPs) regulating membrane dynamics in various cellular processes. These large proteins contain a canonical GTPase domain and various regions that enhance self-assembly via both intra- and inter-molecular interactions [31]. The mitochondrial fission components were first described in yeast genetic screening studies [32]. Dynamin 1 protein (Dnm1) is structurally related to the large dynamin family and was the first protein to have shown a clear role in controlling mitochondrial fission and morphology in Saccharomyces cerevisiae [33,34]. In 1998, Otsuga *et al*. have shown, in yeast, that dynamin-1-like gene (DNML1) mutants, with defects in the predicted GTP-binding domain, had a markedly distorted mitochondrial morphology and an altered network distribution, associated with the impairment of mitochondrial fission [33,34]. Around the same time, the human ortholog of dynamin-1-like protein (DNML1)- DRP1 - was described and was shown to be essential, and the main driver for mitochondrial division in mammalian cells [35,36].

Although DRP1 is described as being mostly a cytoplasmic protein, it has been detected both in cytosol and mitochondria in baseline conditions [19–23]. Indeed, DRP1 translocates to mitochondria upon activation of a stimulus, such as mitochondrial membrane uncoupling, where it links to receptors such as mitochondrial fission factor (MFF) and fission 1 protein (FIS1), constricting the outer mitochondrial membrane in a process dependent on GTPase activity [17]. While MFF is required for DRP1 recruitment, it should be noted that different studies have questioned the role of FIS1 in inducing mitochondrial fission [35–39]. Depending on the cell types and conditions other proteins, such as mitochondrial protein of 18 kDa (MTP18), ganglioside-induced differentiation-associated protein 1 (GDAP1), mitochondrial dynamics protein of 49 kDa and 51 kDa (MiD49 and MiD51), or mitochondrial elongation factor 1 (MIEF1) have a role in cytoplasmically-localized DRP1 activation needed for its recruitment to mitochondria fission sites [38,40,41]. Ganglioside-induced differentiation-associated protein 1 is mainly expressed in neurons and Schwann cells [42]. Additionally, endophilin was reported to act downstream of DRP1 and to be important in the maintenance of mitochondrial morphology [43].

Dynamin-related protein 1 assembles in spirals at sites where endoplasmic reticulum tubules cross over mitochondria and subsequent actin polymerization by inverted formin-2 (INF2) occurs, ultimately leading to mitochondrial fission, as depicted in Figure 1 [44]. Since localization of DRP1 and MFF is dependent on nucleoids, known to be structures composed of both mtDNA and proteins putatively involved in the replication of mtDNA, mitochondrial fission often occurs adjacent to nucleoids [45].

Of note, DRP1 overexpression does not lead to mitochondrial fission per se, since DRP1 activity is dependent on its activation by different post-translational modifications, and on the translocation from cytosol to mitochondria. These modifications may include phosphorylation, SUMOylation, ubiquitination, S-Nitrosylation and O-GlucNAcylation [46–49]. This fact should be kept in mind when interpreting the data described in the literature. The translocation of DRP1 from cytosol to mitochondria may also be impaired by GTPase domain mutations leading to defects in higher-ordered assembly [50]. Several kinases control DRP1 activity by phosphorylation at 3 main sites—Ser616, Ser637 and Ser693 [49,51–56]. The phosphorylation of DRP1S616 can be made by different protein kinases involved in signaling pathways, cell cycle, cell cytoskeleton, or Ca2+ signaling. These include protein kinase C (PKC), CDK1/Cyclin B in the context of mitosis, rho-associated coiled-coil kinase (ROCK) or Ca2+/calmodulin-dependent protein kinase (CAMK -Iα), to promote fission [51,54,57]. On the other hand, phosphorylation of DRP1S637, namely by protein kinase A (PKA), inhibits fission [51–53]. Opposite to this, dephosphorylation of DRP1S637 by calcineurin, which is activated by mitochondrial depolarization and by sustained cytosolic calcium increase, including in situations of starvation and apoptosis stimuli, promotes mitochondrial fission [57]. Finally, phosphorylation of DRP1S693 by glycogen synthase kinase 3β (GSK3β), a negative regulator of glycogenesis and a known regulator of various signaling pathways and cellular functions, has been demonstrated to prevent fission during apoptosis [49]. Several cancer signaling pathways involving PKA, AMP-activated protein kinase (AMPK) and epidermal growth factor receptor-retrovirus associated sequence oncogene signaling pathway (EGFR-RAS) activate DRP1 driven mitochondrial fission, as will be discussed later [19,28,29,58–61]. On the other

hand, after induction of macroautophagy by starvation, mitochondria elongate both in vitro and *in vivo* [62]. Starvation induces an increase in cyclic adenosine monophosphate (cAMP) levels and leads to PKA activation which contributes to a more effective ATP production through mitochondria elongation [63]. For a more in-depth review of the fission and fusion machinery please refer to Silva *et al*. [17].

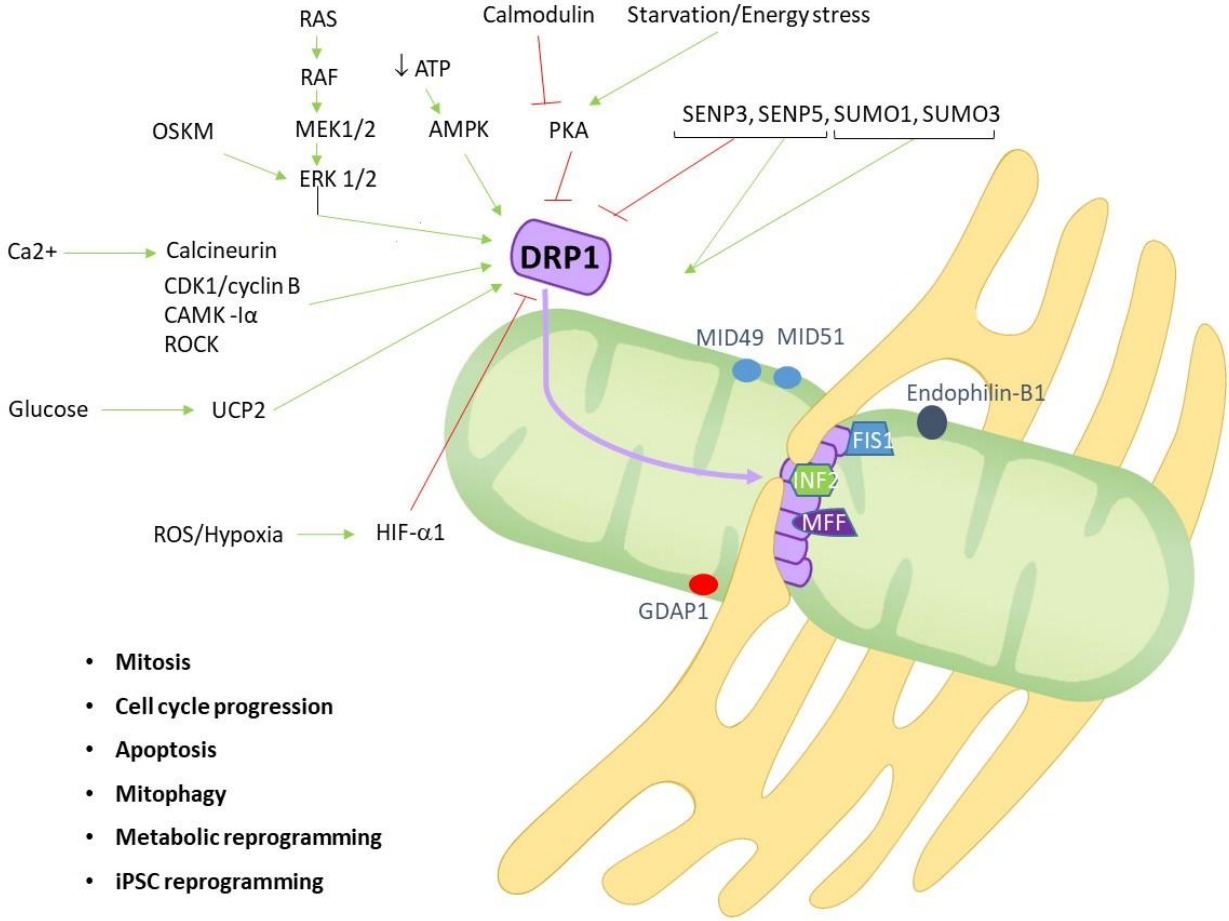

Figure 1. Key players and stimuli in DRP1-mediated mitochondrial fission, both in physiologic and tumor conditions. Green arrows represent stimulation or activation of pathway; red arrows represent repression or inactivation of pathway. SUMO1/Sentrin/SMT3 specific peptidase 3 and 5 (SENP3 and SENP5) and small ubiquitin-like modifier and small ubiquitin-like modifier 1 (SUMO and SUMO1). SENP are deSUMOylating enzymes. For a more in-depth review of the fission and fusion machinery please refer to Silva *et al*. [17].

Dynamin-Related Protein 1 and Cell Death

Mitochondrial division and fusion regulate mitochondrial-dependent intrinsic apoptosis, which relies on the mitochondrial outer membrane permeabilization (MOMP) and in mediators of cell death, such as cytochrome *c*, to be released from the mitochondria [66,75–78]. Mitochondrial fusion protects cells from apoptosis driven by the role of optic atrophy 1 protein (OPA1) in cristae maintenance, which attenuates the MOMP-induced release of cytochrome *c* [79–83]. Mitochondrial fragmentation is known to be involved in several apoptotic models [65]. The role of DRP1 has been detected in complexes with bcl-2-associated X protein (BAX) at mitochondrial fission sites, contributing for the permeabilization of the outer mitochondrial membrane (OMM) and cytochrome *c* release [64]. The role of DRP1 in apoptosis and cell death, as in many other cell biological functions, may seem counterintuitive. Szabadkai *et al*. have used HeLa cells to overexpress DRP1 and thereby assess the role of mitochondrial division in apoptotic signaling and sub-cellular Ca^{2+} homeostasis [65]. The authors have observed a fragmentation of the mitochondrial network, and a blockage of the intramitochondrial Ca^{2+}-propagating waves [65]. However, the apoptotic effect of ceramide on DRP1 expressing cells was significantly reduced, while sensitivity to staurosporine-induced apoptosis was enhanced, raising the hypothesis that a balance between fusion and fission processes may impact on mitochondrial Ca^{2+} responses [65]. In fact, ceramide acts by inducing Ca^{2+} release from the endoplasmic reticulum (ER) and also to sensitize mitochondria to Ca^{2+} impulse, while staurosporine has a direct effect on the OMM permeabilization [65]. Based on these findings, Szabadkai *et al*. proposed a model in which DRP1-mediated mitochondrial fission leads to mitochondria positioning far from the ER, thereby reducing the efficiency of Ca^{2+} uptake, which may still be sufficient for normal mitochondrial function, but may serve as a protective mechanism in responses to stress, preventing apoptosis [64]. Other studies have shown that the downregulation or knock-down of DRP1, or the use of mitochondrial division 1 inhibitor (Mdivi-1), widely used as putative specific DRP1-inhibitor, can prevent cell death and/or promote cell proliferation [22,66,67]. The interpretation of the

data published using this compound should take into consideration the caveat of Mdivi-1 not being currently considered as a specific DRP1 inhibitor, but rather as a weak and reversible complex I inhibitor [84]. In particular, Cassidy *et al*. found that Mdivi-1, retards apoptosis by inhibiting mitochondrial OMM permeabilization and consequently cytochrome c release [66]. Rehman *et al*. have showed that the genetic inhibition, and the use of Mdivi-1, in human lung cancer cell lines led to a decrease in mitochondria fragmentation and a three to four-fold increase in apoptosis [22]. Finally, Yamauchi-Inoue and Oda have demonstrated that DRP1 knockdown in human colon cancer cells resulted in significantly reduced proliferation, increased percentage of cells in sub-G0/G1 cell cycle phase, caspase-3 activation and apoptosis [67]. Interestingly, a reduction in mitochondrial membrane potential was also observed, which may explain the release of cytochrome c seen in apoptosis following caspase activation [67].

All this evidence highlights the potential dual role of DRP1 on cell death and cell proliferation. On one hand, DRP1 may act as a gatekeeper, preventing apoptosis under sub-maximum stress conditions; on the other DRP1-driven mitochondrial fission is needed for cell death and cell proliferation to occur, as explained before. These opposing effects will also become obvious in the tumorigenesis section below, where DRP1 expression or activity may reflect pro-apoptotic or pro-proliferative traits, the former being potentially advantageous for therapeutic purposes.

Dynamin-Related Protein 1 and Metabolic Reprogramming

The relationship between mitochondrial morphology and cell energetics and survival has already been documented. Mitochondrial elongation increases mitochondrial function and protects cells from apoptosis [62,85]. Cells tend to present mitochondria in an elongated form under starvation conditions, and in a fragmented state under a nutrient-rich environment [62,85]. Mitochondrial elongation contributes to mitochondrial function and protects cells from apoptosis under conditions of starvation in mouse embryonic fibroblasts (MEF) cells [62,86].

Mitochondrial metabolic reprogramming is a hallmark of tumorigenesis, and it has been well described that in most of the tumor cell types, an increase in aerobic glycolysis takes place, a phenomenon known as the Warburg effect [87]. However, it is also recognized that cancer cells can adapt their metabolic profile to their needs. A study that shed light on how mitochondrial morphology links with metabolism plasticity in cancer cells was published by Li *et al*., who have investigated the changes in mitochondrial morphology induced by nutrition deprivation in tumor cells, using different tumor type cell lines [70]. A dramatic mitochondrial elongation was induced by starvation. This finding was concomitantly associated with a significant decrease in the DRP1 mitochondrial fraction and a dramatic increase in the phosphorylated form DRP1S637 driven by PKA activation, proven to be required for the energy stress-induced mitochondrial elongation in hepatocellular cell carcinoma (HepCC) cell lines [70]. More importantly, mitochondrial elongation was found to induce a metabolic shift from glycolysis to oxidative phosphorylation during energy stress [70]. Mitochondrial elongation induced by energy stress facilitated cristae formation and the assembly of respiratory chain complexes I–IV to promote oxidative phosphorylation [70]. This, in its turn, led to a negative feedback effect on glycolysis through nicotinamide adenine dinucleotide (NAD+)-dependent sirtuin 1 (SIRT1) activation, a nutrient-sensing deacetylase [70]. Starvation treatment inhibited the acetylation of hypoxia-inducible factor 1α (HIF-1α) and the expression of pyruvate dehydrogenase kinase 1 (PDK1) and lactate dehydrogenase A (LDH-A), which are known to be HIF-1α target genes. This was reversed by the expression of the mutant DRP1S637A, which was associated with mitochondrial fission [70]. This study also indicated that DRP1S637-mediated mitochondrial elongation also predicted a poor prognosis in hepatocellular carcinoma patients [70]. Expression of phosphorylated DRP1S637 was found to be significantly correlated with larger tumor size, high tumor-node metastasis stage, and a significantly reduced overall survival and recurrence free survival [70]. Consistent with these results, nutrient deprivation was associated with OXPHOS/glycolysis interchange in a human glioma cell line, via HIF-1α/cellular myelocytomatosis oncogene protein (c-MYC) pathway, although a correlation with potential changes in mitochondrial shape has not been assessed

in this study [71]. Interestingly, metabolic reprogramming is also a finding that seems to be associated with precancerous lesions of the colon, where a significant increase in gene expression of DNML1 was shown, which was accompanied by indirect markers of the Warburg effect in human samples, as reported by Cruz MD *et al*. [88]. Zou *et al*. have elucidated how DRP1 dysregulation may interact with mitochondrial biogenesis and mitochondrial autophagy (mitophagy), and thereby with metabolic reprogramming. The authors have assessed the autophagic flux by evaluating the impact of autolysosome inhibitors on the microtubule-associated protein-1 light chain 3αphosphatidylethanolamine conjugate (LC3-II) levels, a protein known to be important for autophagosome formation [68]. They have shown a pattern of DRP1 upregulation, which was associated with metabolically less active mitochondria in a breast cancer cell line. This was accompanied by a reduction in the number of mitochondria, an increase of mitochondrial biogenesis markers such as peroxisome proliferator-activated receptor coactivator 1-α (PGC1-α), nuclear respiratory factor 1 (NRF1), and mammalian mitochondrial transcription factor (TFAM), and a significant upregulation of B-cell lymphoma 2 protein (BCL-2) nineteen-kilodalton interacting protein 3 (BNIP3), a mitophagy marker, and of the autophagic flux, suggesting an increased mitophagy that explained the reduced number of mitochondria [68]. This pattern was also confirmed *in vivo* in human breast carcinoma tissue, based on the analyses of a series of human breast cancer from The Cancer Genome Atlas (TCGA database) [68]. Breast cancer cell lines exposed to Mdivi-1 exhibited a reduced autophagic flux and a shift from a glycolytic to an oxidative phenotype, suggesting a reversal of the Warburg effect [68]. The authors suggested a role of DRP1 in the coordinated increase of mitochondrial biogenesis and mitophagy, and in the regulation of breast cancer cell metabolism and survival since a significant decrease of cancer cell viability was also shown. It would be interesting to assess whether these Mdivi-1-induced metabolic effects can be explained by DRP1 inhibition, or through its currently proposed mechanism of action as a reversible Complex I inhibitor [84].

Beyond the effects of starvation in the metabolism of cancer cells, it is also of the utmost relevance to explore the role of hypoxia on metabolic tumor cell adaptation. Using mtDNA-

enriched (SK-N-AS) and depleted (*ρ*0) cells of neuroblastoma cultured in hypoxic conditions, Kuo *et al*. have shown that hypoxia-stimulated HIF-1α expression, which was also influenced by the level of reactive oxygen species (ROS), was accompanied by increases of LDH-A and PDK1 as well as an increased expression

of DRP1 [69]. Additionally, in mtDNA-enriched cells, a higher expression of DRP1 during hypoxia was observed, which was reverted after genetic suppression of HIF-1α [69]. Indeed, mtDNA seemed to be a mediator of HIF-1α, linking metabolic reprogramming to mitochondrial biogenesis [69].

All these data underscore the role of DRP1 as an indirect mediator of a metabolic shift under starvation conditions, when cancer cells need to rely on a more efficient energy production process (OXPHOS) as opposed to the classic glycolytic phenotype. On the other hand, DRP1 should also be seen as a key linking piece that connects different features of the same process (metabolic reprogramming, to meet cell energy needs, mitochondrial biogenesis, building the cell powerhouse that delivers that energy, and mitophagy, a system that promotes the quality control of mitochondria, as will be seen later). Therefore, depending on the different stimuli and needs, and even depending on

specific driver oncogenes, the role of DRP1 is possibly two-pronged: being permissive to OXPHOS or promoting glycolysis.

Dynamin-Related Protein 1 and the Cell Cycle

Mitochondrial fission occurs during cellular division, thus securing a proper mitochondrial number in daughter cells. Dynamin-related protein 1 has been described to be functionally or molecularly linked to Cyclin B, E and D [19,29,54,55,72,73]. As previously mentioned, mitochondrial fission during mitosis depends on translocation of DRP1 to mitochondria and phosphorylation of DRP1S616 by Cyclin B-CDK1 [89]. On the other hand, mitochondrial shape was found to regulate the cell cycle, as demonstrated by the relationship between the mitochondrial hyperfusion at G1-S and the Cyclin E buildup needed to entry into S phase [89].

Additionally, DRP1 has been identified as one of the Cyclin D1-interacting proteins in human tumors, including breast and colorectal cancer [89]. Previous studies have demonstrated that DRP1-driven mitochondrial fission is critical for regulation of cell proliferation in a Drosophila model system, as well as in mammalian cells [89]. Mitochondrial function can impact cell cycle regulation; however, this has been an underexplored area in cancer research.

Taguchi *et al*. have studied mitochondrial dynamics and inheritance in mammalian cells undergoing mitosis in vitro and they showed that mitochondria have a tubular network appearance in interphase cells, moving into fragmented status in early mitotic stage, and going back to filamentous structures in the late phase of mitosis, the mitochondrial fission being a result of DRP1S585 phosphorylation by CDK1/Cyclin B [54]. Although the exact mechanism by which fission occurs is not yet totally known, endophilin and probably other downstream factors may play a role [90].

The elongated shape of mitochondria in G1/S interface is linked to the cellular requirement of ATP and high Cyclin E levels [29,72]. It is therefore thought that throughout the cell cycle, mitochondrial dynamics provides the energy requirements that are needed.

Parone *et al*. showed that downregulation of DRP1 in HeLa cell lines causes mitochondrial dysfunction, with an increase in ROS levels, a loss of mtDNA, a reduction in cellular ATP, proliferation arrest, and autophagy [91]. It seems therefore that cellular homeostasis is dependent on DRP1-dependent mitochondrial fission. On the other hand, mitochondrial hyperfusion induced by DRP1 deficiency was found to trigger a signaling of replicative stress by which ataxia-telangiectasia mutated/checkpoint kinases 2 and 1 (ATM/Chk2 and ATR/Chk1) DNA damage signaling pathways, as well as the ATM kinase-dependent G2/M cell cycle checkpoint, are activated [72]. A pattern of genomic instability and aneuploidy in p53 wild type and mutated cells, independent of ATP production defects or ROS production, was also found, suggesting that DRP1 may be implicated in mitochondria-nucleus retrograde signaling and raising the hypothesis that mitochondria play a role in tumorigenesis [72].

Rehman *et al*. have compared the level of mitochondria fragmentation in several human lung cancer cell lines and normal human cell lines. They observed that all malignant cells presented

a markedly higher level of mitochondria fragmentation, which was linked to higher DRP1 and lower mitofusin-2 (MFN2) expression levels, the latter being a protein involved in mitochondrial fusion [22]. The same was observed in lung adenocarcinoma samples, when compared to adjacent normal lung tissue. Additionally, the levels of phosphorylated DRP1S616 were also significantly higher, as opposed to phosphorylated DRP1S637 which was lower in both lines. The genetic inhibition of DRP1, and the use of Mdivi-1, has led to a decrease in mitochondrial membrane potential, and a decrease in the number of cells in S phase and an increase in number of cells in G2 phase, again indicating an inhibition of the mitotic program [22]. Both these interventions were also tested in a lung adenocarcinoma xenograft model, leading to a significant decrease in tumor size [22].

Mitra *et al*. have reported a relationship between mitochondrial morphology and cell cycle control at the G1–S boundary [29]. Mitochondria change from fragmented structures into a hyperfused state at G1–S transition. In this stage of the cell cycle, the mitochondrial network presents a greater ATP output than isolated mitochondria at any other cell cycle stage. Hyperfused mitochondria might also play a role in tumorigenesis, since it is known that many cancer cells present dysregulated Cyclin E levels, the cyclin responsible for G1-to-S phase progression and lose control of G1–S transition [92,93].

Zhan *et al*. have shown that the expression of DRP1 increased mitochondrial fission and promoted the proliferation of HCC cells both in vitro and *in vivo*, by enhancing the G1/S phase transition [94]. Additionally, the authors have verified that DRP1 knockdown induced a significant G1 phase arrest in vitro, and reduced tumor growth *in vivo* [94]. More importantly, they have demonstrated that the promotion of proliferation by DRP1-mediated mitochondrial fission was mediated through p53/p21 and nuclear factor kappa B (NF-kB) /cyclins pathways [94].

Finally, Tanwar *et al*. have recently published an exploratory analysis of gene expression data from the 31 cancer types in TCGA, showing that DRP1 is predominantly co-expressed with genes involved in the cell cycle, and in gene expression and metabolism, across the majority of the cancer types [74]. In particular, their investigation on epithelial ovarian cancer (EOC)

revealed that DRP1 co-expresses with the cell-cycle module responsible for mitotic transition, which included over 70 genes involved in various phases of cell cycle (G1 phase, G1/S transition, S phase, G2/M transition and M), such as the mitotic transcription factor forkhead box M1(FoxM1) and its key downstream targets regulating mitotic transition. Inactivation of DRP1 through DRP1 knock-down in EOC cells led to attenuation in mitotic transition [74]. Interestingly, DRP1-cell-cycle co-expression module was detected in epithelial ovarian tumors which responded to chemotherapy, suggesting that DRP1 driven mitosis may contribute to chemo-sensitivity of the primary tumors.

In summary, the pattern of higher DRP1 expression observed in different malignant tumors, as we will later see, seem to indicate a higher proliferative profile of those cells. Complementary to this, DRP1 could also represent a caretaker mechanism, in the sense that its downregulation can trigger the activation of DNA damage signaling pathways, and in an extreme context, ultimately lead to tumorigenesis. The fact that DRP1 is directly involved in cell cycle progression makes it an attractive target for directing therapy agents that interfere with cell proliferation.

Dynamin-Related Protein 1 Expression and its Role in Tumorigenesis

DRP1 expression patterns and its role in cancer have been documented in several tumor models and are summarized in Table 2. Wieder et al. described an expression of phosphorylated DRP1S616 in nearly half of the cases of a melanoma series, 95.6% of which were *BRAF*^{V600E} tumors [19]. Interestingly, the same relationship with B-Raf proto-oncogene (*BRAF*) status was observed in dysplastic nevi, with 92% of *BRAF*^{V600E} samples being positive for phosphorylated DRP1S616 [95]. Genetic inhibition of DRP1 in *BRAF*^{V600E} melanoma cell line led to a loss of expression of DRP1 that was correlated with decreased cell proliferation. On the other hand, the use of Mdivi-1 led to a decrease in DRP1-dependent mitochondrial fission and dose-dependent apoptosis, which was not seen in the wild type (WT) *BRAF*^{WT} melanoma cell line, suggesting that the induction of phosphorylated DRP1S616 in dysplastic

nevi and in primary melanoma may be a contributing factor to *BRAF*^{V600E} disease, raising the question of its potential role as a prognosis biomarker in this context [95]. These results should take into consideration the caveat of Mdivi-1 not being currently considered a specific DRP1 inhibitor [84,95].

Rehman *et al*. documented an increase in DRP1 expression in tissue samples from patients with lung adenocarcinoma [22]. An identical pattern was observed in cultured lung cancer cell lines, with increased levels of phosphorylated DRP1S616 and decreased levels of phosphorylated DRP1S637 [22]. Interestingly, Mdivi-1 was tested in a lung adenocarcinoma xenograft model and proved to significantly reduce tumor size, with an increase in the uptake of 18F-fluorodeoxyglucose (18FDG) in the residual tumor, suggesting an effect on tumor metabolism [22]. Considering the currently proposed mechanism of action of Mdivi-1, as an inhibitor of complex-I and ROS production, it would be interesting to assess if the described reduction of tumor size may be related with potential changes in mitochondrial metabolism.

Ferreira-da-Silva *et al*. studied benign and malign thyroid tumors, including oncocytomas, which are characterized by a large accumulation of mitochondria in the cytoplasm of their cells [23,99,100]. Interestingly, they found a statistically significant overexpression of DRP1 protein in the oncocytic versus the non-oncocytic thyroid tumors. This pattern was also found when they compared oncocytic carcinomas with oncocytic adenomas [23]. However, the same trend was not observed when comparing benign and malignant tumors overall, nor within the non-oncocytic group of adenomas versus carcinomas. Following these same findings, Ferreira-da-Silva *et al*. have documented a statistically significant higher expression of DRP1 in an oncocytic thyroid carcinoma cell line compared with a non-oncocytic cell line, an observation that was not explained by differences in mRNA expression [23]. The higher expression of DRP1 was also associated with a more fragmented mitochondrial network [23]. The genetic inhibition of DRP1 reduced cell motility in the oncocytic cell line by close to 50%, a pattern that was also seen with the use of Mdivi-1 [23]. The higher DRP1 expression and fission profile may explain the oncocytic pattern of this particular subset of thyroid tumors, given the known role of DRP1 in mitochondrial biogenesis [23,101]. The association between DRP1 and the

potential for higher migration and invasion capacities of the malignant oncocytic tumors is a trait that may also be explained by DRP1 overexpression, and one that has been shown in other tumor models, as later described [23].

Serasinghe *et al*. have shown that E1A plus RASG12V-infected MEFs induce DRP1 mRNA expression, DRP1 expression, its activation through phosphorylation of serine 952 residue (murine equivalent of DRP1S616 phosphorylation), and a glycolytic phenotype [19]. Through DRP1 genetic inhibition, and also after the use of Mdivi-1, DRP1 expression and function were found to be required for MAPK/ERK kinase (MEK) triggered transformation when RASG12V signaling is induced [19]. When they tested two small MEK inhibitors in those transformed cells this led to increased mitochondrial fusion, which was shown to be directly related to the phosphorylation of DRP1S592 [19]. Similar results were observed in a human *BRAF*^{V600E} melanoma cell line, where different upstream and downstream mitogen-activated protein kinase (MAPK) inhibitors have led to mitochondrial fusion, which seemed to be dependent on direct effects in the MAPK pathway, since drug-resistant cell lines were not sensitive to this effect [19]. This result seemed to be independent of mitochondrial biogenesis and was reversible [19]. Similarly, MAPK inhibitors inhibited DRP1 mRNA, protein, and DRP1S616 phosphorylation, and led to reprogramming of mitochondrial metabolic function, shifting it to an OXPHOS patterned metabolism [19]. These authors also documented a significantly higher phosphorylated DRP1S616 expression rate in *BRAF*^{V600E} melanoma patient samples when compared with *BRAF*^{WT} samples [19]. According to Serasinghe *et al*. experiments, DRP1 seems to regulate mitochondrial function before an oncogenic signaling is initiated, during carcinogenesis and after oncogenic MAPK signaling inhibition [19].

Lennon *et al*. have specifically explored mitochondrial morphology through fractal dimension and lacunarity measurements in mesothelioma cell lines, as a prediction of responses to treatments that interfere with mitochondrial metabolism [102]. Fractal dimension and lacunarity are quantitative measurements which allow the description of complex structures, such as mitochondria. The former relies on a mathematical principle of self-similarity between different biological structures, while the latter is based on the texture of a shape. An altered

ratio of DRP1-MFN2 in both total cell lysates and mitochondrial fraction was detected, suggesting a higher relative rate of fission as compared to fusion [102]. Interestingly, mitochondrial morphology showed a better correlation with mitochondrial inhibitors sensitivity than did metabolic function [102]. As pointed out by the authors, increased fission seemed to be associated with decreased mitochondrial activity and mitochondrial membrane potential, which could explain an increase in cell death with mitochondrial inhibitors [102].

Hagenbuchner *et al*. have studied the mitochondrial effects of Survivin, a known anti-apoptotic protein that is overexpressed in neuroblastoma with gain of chromosome 17q, typically associated with high stage cancer, poor prognosis, and chemotherapy resistance [97]. In Survivin-expressing cells, mitochondria presented as punctuated, perinuclear structures, due to an increase in the expression of DRP1, which was accompanied by a reduction in the expression of BCL-2-like protein 11 (BIM) [97].

In these cells, DRP1 localized in mitochondria, but no cytochrome c release was observed due to the absence of BIM [97]. These effects were affected through genetic inhibition of DRP1, and also after the used of Mdivi-1 [97]. Curiously, an effect of Survivin on oxidative phosphorylation, through an impact on complex I and IV, was also shown to result from DRP1-induced mitochondrial fission, with no changes in ATP levels, raising the hypothesis that ATP in these Survivin expressing cells may be produced as a result of glycolysis, which was supported by the increase in glucose consumption and lactate production, and by the effect that glycolysis inhibitors had on cell viability reduction and sensitivity to chemotherapy agents [97].

Recently, Guerra *et al*. have documented an increase in the expression of DRP1 and BNIP3, a molecular mediator which promotes mitophagy, the antioxidant augmenter of liver regeneration (ALR), and the anti-apoptotic molecule BCL-2 in cancer cells of type I endometrial carcinoma with previously described alterations in respiratory complex I (oncocytic-like phenotype), as compared to matched non-malignant tissue and hyperplastic tissue, linking mitochondrial dysfunction to the expression of pro-fission, anti-oxidant, and anti-apoptotic proteins [24].

Tanwar *et al*. conducted experiments of downregulation of DRP1 in a human ovarian carcinoma cell line, showing a potential causal role of DRP1 in mitotic transition and cell proliferation in EOC cells [74]. These authors have also compared the expression of aldehyde dehydrogenase 1A1 (Aldh1A1), a marker for ovarian cancer stem cells, between primary and relapse tumor samples and have found an inverse relationship between Aldh1A1 and DRP1 expression [74]. This finding suggests that the modulation of DRP1 may potentially be involved in the stem cell properties of the relapsed EOC disease [74]. Based on their results, DRP1 seems to associate with cell cycle acceleration in some relapsed resistant patients (DRP1-High) as compared to others (DRP1-Low) where this does not seem to happen. The authors thereby hypothesize that DRP1 may have a pro-apoptotic role in DRP1-Low and an anti-apoptotic role in DRP1-High patients [74]. Additionally, they have suggested that a DRP1-based-gene expression-signature from primary tumors could stratify patients for survival after exposure to chemotherapy, since the pattern of genes expression seems to differ in both DRP1-High and DRP-Low groups [74].

The RAS-activated molecule recombinant protein of human ralA binding protein 1 (RALBP1) regulates the effect of Cyclin B1 on DRP1 [54,55]. Although RAS-ERK signaling-driven regulation of DRP1 contributes to cell transformation, as previously mentioned, no relationship with cell cycle alteration was found [19,59]. Various studies have implicated extracellular signal-regulated kinase 1 and 2 (ERK1/2) in regulating DRP1 function (Figure 1). Yu *et al*. have shown that ERK1 could phosphorylate DRP1 in vitro [103]. Gan *et al*. studied the oxidative stress responses in cytoplasmic hybrid (cybrid) derivatives of neuronal cells, incorporating platelet mitochondria from AD [104].

They showed that ERK1/2 activation driven by oxidative stress increased DRP1 expression and its recruitment to mitochondria, generating increased fission in AD cybrids [104]. However, no functional link between ERK and DRP1 was established [104]. As mentioned previously, Serasinghe *et al*. have demonstrated that DRP1S616 is phosphorylated by ERK1/2 in cancer cells, promoting mitochondrial fission to support RAS-dependent transformation and tumor growth [19]. When this phosphorylation was reverted in vitro, cells have undergone apoptosis

[19]. Recently, Kashatus *et al*. showed that the expression of mutant RAS in HEK cells promoted DRP1-dependent mitochondrial fragmentation [61]. Additionally, knockdown of DNM1 inhibited the growth of transformed cell tumor xenografts [61]. ERK2 and activated RAS, RAF or MEK mutants were shown to phosphorylate human DRP1S616 in vitro, an effect that was abolished by MEK inhibitors [61]. This was accompanied by a reversal of the mitochondrial fission [61]. ERK1/2-dependent DRP1 phosphorylation and mitochondrial fission have been described to induce pluripotent stem cells (iPSCs) during the reprogramming of somatic cells [105]. Prieto *et al*. have shown that cellular reprogramming into iPSC induced mitochondrial fission early in this process, which was dependent on DRP1 and accompanied by an increase in DRP1 phosphorylation at the murine equivalent of human DRP1S616, with kinetics matching DRP1 recruitment to mitochondria [106]. It was also shown that mitochondrial fission was inhibited by a MEK inhibitor, a pattern which was reverted by a DRP1 phosphomimetic mutant. This raised the hypothesis that ERK signaling may be required for mitochondrial fission early in the reprogramming process [106].

Morita M. *et al*. have shown that the nutrient-sensing mechanistic/mammalian target of rapamycin complex 1 (mTORC1), which is known to be activated in many different malignant tumors, stimulates the translation of mitochondrial fission process 1 (MTFP1) protein [107]. MTFP1 is, in its turn, associated with phosphorylation and mitochondrial recruitment of DRP1 and a mitochondrial fission pattern [107]. Interestingly, they have found that the suppression of mTORC1 activity led to increased mitochondrial fusion due to the reduced translation of MTFP1, which is mediated by translation initiation factor 4E (eIF4E)- binding proteins (4E-BPs) [107]. The authors further concluded that uncoupling MTFP1 levels from the TORC1/4E-BP pathway after mTOR inhibition blocks the hyperfusion status and leads to apoptosis, thereby offering a new therapeutic opportunity for these type of anti-cancer drugs, converting them from cytostatic to cytotoxic [107].

The mitochondrial uncoupling protein 2 (UCP2) also seems to control mitochondrial fission through DRP1 expression regulation [108,109]. Toda *et al*. reported mitochondrial changes, such as increase in mitochondrial density and reduction in mitochondrial size, in ventromedial

nucleus of the hypothalamus (VMH) neurons mediated by UCP2, suggesting that UCP2 is involved in the regulation of the mitochondrial fission process [110]. In this way, Toda *et al*. assessed the effect of UCP2 in DRP1 in response to a glucose load and verified a significant increased ratio of phosphorylated DRP1/DRP1 in UCP2 knockout mice with selective re-expression of UCP2 [110]. Interestingly, a few years ago, UCP2 was found to be overexpressed in Hürthle cell tumors [111]. These findings may partially explain the pattern of DRP1 overexpression observed by Silva *et al*. in Hürthle cell tumors of the thyroid, known to be characterized by at least 75% of oxyphilic cells [23].

Role of Mitochondrial Dynamics in Invasion and Metastization

In a series of human breast cancer samples, Zhao *et al*. observed a significantly increased expression of DRP1 protein in in situ ductal carcinoma in comparison with normal tissue, and in invasive breast cancer and lymph node metastases in comparison with in situ ductal carcinoma [21]. The authors also reported an increased expression of DRP1 and phosphorylated DRP1S616 in metastatic breast cancer cell lines, as compared to non-metastatic breast cancer cell lines [21]. DRP1 genetic inhibition led to reduced migration and invasion capacities, which was also verified for cell migration when pharmacological inhibition with Mdivi-1 was tested [21]. Cell cycle or cell viability did not seem to be affected by DRP1 changes [21]. Interestingly, DRP1 silencing led to reduced cell spreading and lamellipodia formation, typically seen in the edge of migrating cells, which was accompanied by a change in mitochondria distribution within the cell, from perinuclear to a more scattered state, independent of the membrane potential [21]. The aforementioned findings suggested that the upregulation of DRP1 may be an early event in invasive breast cancer development, and formation of lamellipodia is dependent of mitochondria fission [21].

It was demonstrated in a glioblastoma in vitro model that hypoxia induces upregulation of DRP1, mitochondrial fission and cell migration [112–115]. Following these observations, Han

et al. looked at the effect of hypoxia in breast cancer cell migration driven by mitochondrial dynamics [20].

Besides the similar pattern of DRP1 expression in metastatic breast cancer cell lines documented before, Han *et al*. showed that hypoxia led to mitochondrial fission and to a significantly increase in migration of the metastatic cell line in comparison with the non-metastatic cell line. The genetic inhibition of DRP1, as well as the used of Mdivi-1, led to a significant reduction in mitochondrial fission as well as in hypoxia-induced migration [20]. At variance with the non-metastatic cell line, treatment with cisplatin (CDDP) induced apoptosis, mitochondrial fission, increase in intracellular levels of ROS and a decrease in metalloproteinase (MMP) in the metastatic cell line, which was reverted by the inhibition of DRP1 [96]. These results indicate that mitochondrial fission driven by DRP1 induces the metastatic cell line to become more sensitive to cisplatin in hypoxic conditions, potentially but not only through the impact on intracellular ROS and MMP, an effect that was not observed in the non-metastatic cell line [96].

Finally, a study that has shed some light onto the mechanisms that link cell motility and migration with mitochondria and OXPHOS dysfunction, has been published by our group [116]. We have shown that cybrid cells harboring a specific mtDNA mutation are less prone to apoptosis, have a higher motility and migration ability, and produce larger tumors and more lung metastases in a mouse model in comparison with wild-type cells [116].

Future Perspectives and Clinical Implications

The role of DRP1 in key hallmarks of cancer, as cell proliferation and survival, apoptosis failure, metabolic reprogramming, invasion and metastization, and even insensitivity to anti-growth or anti-proliferative signals, depends most likely from the interplay between microenvironment stimuli, cells' genetic background, cytotoxic or targeted treatment strategies, and the tumor cell's continuous adaptation to all of these factors. In other words, we may look at DRP1 as a key molecular link between several biological cell processes, which

acts as a key player in the plasticity of tumoral cells under various internal and external contexts (Figure 2). This concept has implications both on the interpretation of its biological significance at any given moment of the tumorigenesis process, as well as on the potential effects of its inhibition which can also be paradoxical. As an example, Szabadaki *et al*. have shown that DRP1 overexpression can prevent apoptosis, but it had a negative effect on cancer survival following MAPK inhibitors [19]. There is evidence suggesting that some tumor cells may become dependent on ERK1/2-driven DRP1 phosphorylation, thus indicating that DRP1 inhibition may be a potential therapeutic strategy for such tumors [104]. Others, however, have demonstrated that DRP1 inhibition can prevent cell death and promote proliferation [29,65,66]. Some of the research presented in this revision suggest a new concept, in which mitochondrial-targeted cancer therapy could be additive to or synergized with therapies that address cancer cell proliferation, such as promoting mitochondrial glucose oxidation [19].

It remains important that the link between DRP1 and cell cycle is better understood. Mitra *et al*. have found that the G1–S transition and Cyclin E levels can be regulated by the mitochondrial state, thereby opening new areas of exploration relating mitochondria with cancer [29]. Zou *et al*. have stressed the emerging evidence of PGC1-α contributing to tumor growth, and therefore have proposed the critical importance to target both mitochondrial biogenesis and mitophagy for effective cancer treatment, a concept to be tested in future research as a means to test effectiveness for breast cancer treatment [68]. Additionally, the definition of a relationship between HIF-1α and DRP1 may be of relevance to assess its clinical applications in the future [71].

Finally, we believe it is worthwhile to stress the research recently published by Tanwar *et al*. [74]. Their DRP1-based analysis highlights that DRP1-driven cell cycle regulation is present in several cancer types, which may allow response to therapies targeting proliferating cells [74]. In particular, their results point out to an important role of mitochondria in ovarian cancer chemo-resistance and relapse [74].

To address the issue on how DRP1 can be targeted, it is important to highlight that, although Mdivi-1 has been widely used as a putative DRP1 inhibitor in vitro and *in vivo*, including in

much of the published data referenced in this review, a recent report has proposed an alternative mechanism of action for this compound, as a reversible mitochondrial complex I inhibitor, not impairing Drp1 GTPase activity. Targeting DRP1 in the context of cancer still seems a promising approach, but not without the challenges of designing and developing compounds that specifically inhibit GTPase

activity, and of the complex interplay between mitochondria dynamics and cell requirements in every stage of tumorigenesis.

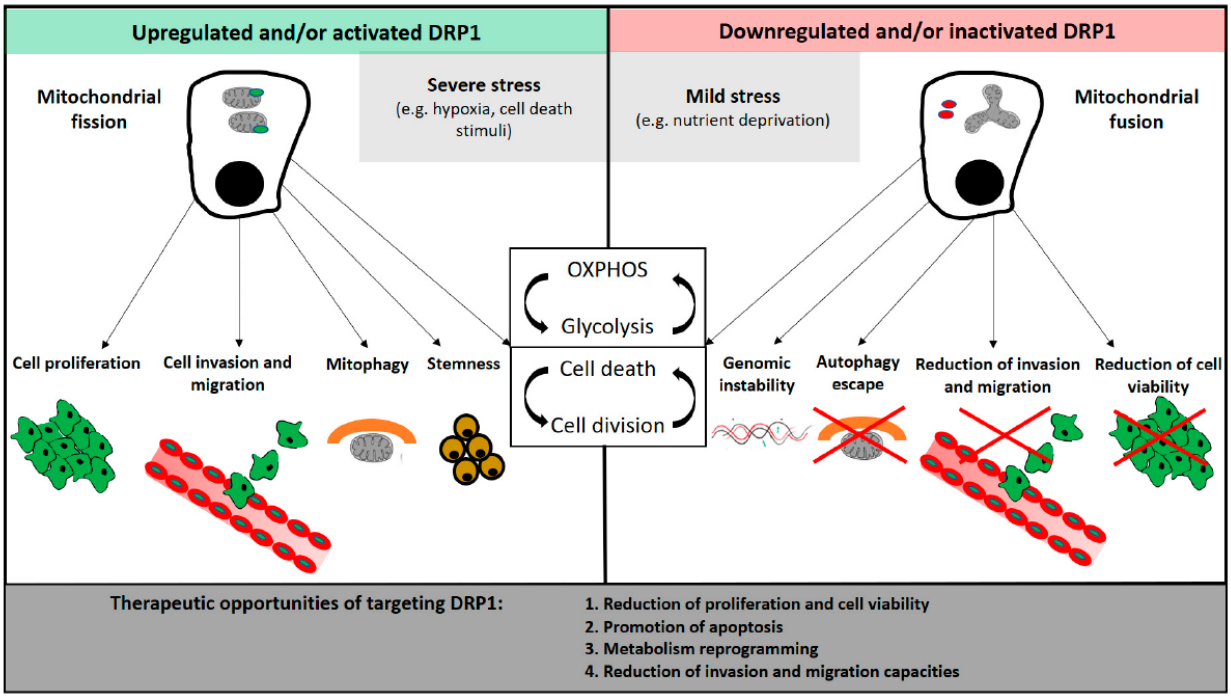

Figure 2. Effects of DRP1 activation and/or upregulation, and associated mitochondrial fission patterns, on tumorigenesis. Inactivation and/or downregulation of DRP1 may have a counteracting effect on tumorigenesis, which could be used as a therapeutic approach in cancer. The effects of both DRP1 activation and inactivation on metabolism reprogramming, and on cell cycle and cell death, should be seen as a continuously dynamic adaptive mechanism to internal and external challenges.

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2. Objectives

With this project we intended to characterize the expression of DRP1 in a large series of FCDTC, aiming to explore if it can serve as a prognostic and/or predictive molecular biomarker in the clinical management of TC.

We also aimed to explore the consequences of the pharmacological inhibition of DRP, alone or in combination with RET/PTC - RAS - BRAF - MEK - ERK signalling pathway inhibition, on key hallmarks of cancers, such as cell viability, apoptosis, cell cycle as well as on the expression of sodium/iodide symporter (NIS).

With this, we wished to postulate new potential treatment strategies including DRP1 as a target, with the objective of delaying or overcoming resistance to currently standard treatment based on RAI and TKIs.

3. Materials and Methods

To achieve our objectives, we have:

1. Characterized the expression of DRP1 based on a series of 259 cases of FCDTC - including 253 cases of DTC and 7 cases of PDTC - already described from a clinico-pathological and genetic perspective, and correlated with this characteristics and with clinical outcomes, such as disease-free status at the end of follow-up, disease-specific survival and overall survival;

2. Characterized the DRP1 expression *in vitro* based on a set of various thyroid cancer derived cell lines known to have different molecular and genotypic profiles (113):

    - TPC1 cell line - derived from a PTC and harbouring the *RET/PTC1* rearrangement; this cell line was obtained from National Cancer Center Research Institute, Tokyo, Japan, 1989, and was ceded by Dumont JE and Mareel M;
    - 8505C cell line - derived from a UTC and harbouring the *BRAF*^{V600E} mutation; this cell line was obtained from Electro-Chemical and Cancer Institute, Chofu, Tokyo, Japan, 1993, and was ceded by Mareel M;
    - C643 cell line - derived from a UTC and harbouring the *H-RAS G13R* mutation; this cell line was obtained from Institute of Anatomy and Cell Biology, Goteborg University, Goteborg, Sweden, and was ceded by Mareel M;
    - XTC-1 cell line - derived from a FTC (breast metastasis) and harbouring the Del T (67619) *PTEN* frameshift mutation; this cell line was obtained from Surgery Service, Veterans Affairs Medical Center, San Francisco, California, 1996, and was ceded by Wong MG and Savagner F.

3. Documented the effect of the *in vitro* pharmacological inhibition of DRP1 and BRAF using a putative selective inhibitor of DRP1, Mdivi-1 and a BRAF inhibitor, alone or in combination, on:
    i. Cell viability
    ii. Apoptosis
    iii. Cell cycle
    iv. MAPK and DRP1 signalling pathway molecular targets
    v. NIS expression

in the aforementioned cell lines.

3.1 Tumor samples and Immunochemistry (IHC)

We have studied 259 formalin-fixed, paraffin-embedded human tissue samples from FCDTC collected from the biobank of the Institute of Molecular Pathology and Immunology of the University of Porto (Ipatimup). The material used in this study originated from patients followed in two University Hospitals in Portugal - *Centro Hospitalar São João* (CHSJ) and *Centro Hospitalar e Universitário de Coimbra* and from the clinical databases of these hospitals. This work was approved by the local Ethics Committee for Health (CES), and all the procedures described in this study followed the national legal requirements and the Helsinki declaration.

The demographic and clinicopathological data of the patients were retrospectively collected from the histopathological reports and clinical databases. The histology of all tumor samples was reviewed independently by two pathologists, and the thyroid tumor classification was performed according to the WHO criteria (15). Patients were stratified by clinicopathological characteristics in the following categories: gender, age (≥45 years or <45 years), histological diagnosis, TNM (114) stage, tumor size, tumor capsule invasion, vascular invasion, thyroid capsule invasion, extrathyroidal invasion, multifocality, presence of lymph node and distant metastases, presence of $BRAF^{V600E}$ mutations, presence of *TERT* promoter

mutations (-124G>A and -146G>A), cumulative dosage of radioiodine treatment, persistence of the disease at the end of follow-up, disease-specific mortality, and overall mortality. «Aggressive variants» were defined as all cases of solid/trabecular, diffuse sclerosing, tall cell or columnar cell PTC, as well as all cases of PDTC. When comparing PTC with FTC and FVPTC, «aggressive variants» were excluded. We performed an analysis for the whole sample and a subanalysis for the major histotypes. Considering that FTC and FVPTC share morphological (follicular pattern) and molecular features (high proportion of *RAS* mutations), we also considered a subgroup encompassing these two types of tumors (115).

Immunohistochemical analysis

Immunohistochemistry (IHC) was performed in 3-µm formalin-fixed, paraffin-embedded sections. Sections were deparaffinized and rehydrated in a series of decreasing concentrations of ethanol solutions. Deparaffinized sections were subject to heat-induced antigen retrieval in 1 mM pH 9.0 ethylenediaminetetraacetic acid buffer (EDTA) (LabVision Corporation, Fremont, CA, USA). Endogenous peroxidase activity was blocked with UltraVision Hydrogen Peroxide Block and non-specific bind was blocked using UltraVision Block reagent from UltraVision Quanto Detection System HRP DAB (Thermo Scientific/Lab Vision, Fremont, USA) for 10 minutes. The sections were then incubated in a humidified chamber, according to the manufacturer's specifications, with the following primary antibodies: mouse monoclonal antibody for DRP1 (1:100) ref. 611112 (BD Biosciences), rabbit polyclonal antibody for S616-p-DRP1 (1:500) ref. 3455, from Cell Signaling. The sections were then washed and stained by using the UltraVision Quanto Detection System HRP DAB (Thermo Scientific/Lab Vision, Fremont, USA). All sections were counterstained with Mayer's hematoxylin. Positive controls from previously tested kidney samples were used in every run. To assess the specificity of the immunostaining, tumor sections not incubated with the primary antibody were used as negative controls. Whenever present, scattered macrophages and muscular tissue were considered as internal positive controls for both proteins. A second

internal and positive control for each tumor sample was the expression of both proteins in normal adjacent thyroid tissue.

Evaluation of immunohistochemical staining

Immunostaining was semi-quantitatively evaluated by three observers without the knowledge of any clinical information of the cases. The expression of DRP1 in tumor tissue was evaluated according to an immune-reactive staining score (IRS) adapted from other studies (116-118). Immunohistochemical positivity was defined as cytoplasmic staining for DRP1 and S616-p-DRP1, and immunostaining scores were based on the intensity and the extension of tumor cells immunostaining, as described in Table 2. A total IRS was then obtained by multiplying the intensity (I) and extension (E) scores, i.e. IRS = I x E, ranging from 0 to 12. Positive expression or overexpression of DRP1 and S616-p-DRP1 in TC sections was defined by an IRS of 6 or higher. This positivity criterion was based on previous observations that the immunostaining of DRP1 in normal adjacent thyroid tissue was usually weaker than in neoplastic tissue with an IRS score of 4 or lower.

3.2 Thyroid Cell Lines and Cell Culture

All cell lines were maintained at 37º C, in a humified atmosphere, 5% CO2. TPC1, C643 and 8505C were maintained in RPMI (Roswell Park Memorial Institute) 1640 medium (RPMI-STA, Labclinics, Barcelona, Spain), supplemented with 10% fetal bovine serum (FBS) (LTI10500064, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and 1% (v/v) penicillin-streptomycin (L0022100, LabClinics, Barcelona, Spain). XTC-1 was cultured in Dulbecco's modified Eagle's medium (DMEM) F12 (L0090-50, LabClinics, Barcelona, Spain) again supplemented with 10% FBS and 1% (v/v) penicillin-streptomycin.

3.3 Treatment of Thyroid Carcinoma Cell Lines with Mdivi-1 and Dabrafenib

All cell lines were treated with Mdivi-1 and Dabrafenib alone and in combination. Mdivi-1 (M0199, Sigma-Aldrich, St. Louis, Missouri, USA) and GSK2118436 (Dabrafenib) (S2807, Selleckchen, Houston, Texas, USA) were dissolved in dimethyl sulfoxide (DMSO). Drugs were added to the cell culture media and incubated for 48 and 72 hours. DMSO was used as control.

3.3.1 Cell Viability Assays

Cells were plated in 96-well plates with a density of 2×10^3 and 3.5×10^3 cells/well for TPC1 and XTC-1, C643 and 8505C, respectively, in 100 µL of their corresponding medium. After 24 h cells were treated, by adding 100 µL of media with dissolved drugs at the desired concentrations. We have done Presto Blue and Sulforhodamine B Assay assays for all cell lines, but we chose to present only the Sulforhodamine B Assay results. All cell lines were treated with Mdivi-1 in the following concentrations: 12.5, 25 and 50 µM or with Dabrafenib in the following concentrations: 2.5, 10 and 15 µM. Finally, for Sulforhodamine B Assay cells were treated, with Mdivi-1 25 µM plus Dabrafenib 2.5 µM and Mdivi-1 25 µM plus Dabrafenib 10 µM, for the combined treatment. Cells were incubated for 48 and 72 hours, in the culture conditions already referred.

3.5 Apoptosis and Cell Cycle

After trypsinization, cells were plated in 6-well plates with a density of 1×105, 1.5×105 and 2×105 cells/well for TPC1, 8505C and XTC-1, and C643, respectively. The cell treatments included those designated in Table 2, initiated 24 hours after cell seeding. The conditions comprised treatments with each drug and both drugs combined during 48 and 72 hours.

3.5.1 Apoptosis

For apoptosis analysis, the harvested cells were incubated, in the dark, with 2.5 % of annexin V (31490013x2, Immunotools, Friesoythe, Germany), for 10 minutes, and 50 µg/mL of propidium iodine (PI) (P4864, Sigma-Aldrich, St. Louis, Missouri, USA), for additional 5 minutes. Annexin V binds to the phosphatidylserine expressed on the plasma membrane of apoptotic cells, whereas PI is a dye that only penetrates the unviable membrane of necrotic cells. Cells were analyzed by flow cytometry in the BD Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, New Jersey, USA), counting 20 000 events for each sample. The cells autofluorescence was also measured. Three experiments were performed for each cell line and condition. Data was obtained and analyzed using the BD Accuri C6 Software (BD Biosciences, Franklin Lakes, New Jersey, USA).

3.5.2 Cell Cycle

For cell cycle analysis, the harvested cells were fixed and incubated overnight with ice-cold 70% ethanol. Fixed cells were resuspended in 200 µL of DNA staining solution comprising phosphate buffered saline (PBS) (containing 0.01 M Na2HPO4, 0.0018 M KH2PO4, 0.1370 M NaCl, 0.0027KCl, pH 7.4) 1x, 100 µg/mL of RNase A (Thermo Fisher Scientific, Darmstadt, Germany) and 5 µg/mL of PI. Cell staining was measured by the flow cytometer, counting 20000 events per sample. Three experiments were conducted to each cell line and condition. Cell cycle results were analyzed using the FlowJo 7.6.5 Software (Tree Star, Inc., Ashland USA).

3.6 Protein Expression

For protein expression, cells were plated in 6-well plates with a density of 1×10^5, 1.5×10^5 and 2×10^5 cells/well for TPC1, 8505C and XTC-1, and C643, respectively. 24 hours

after seeding, cells were incubated in the conditions described in Table 2 for 48h. At the end of this timepoint, cells were lysed with a reagent-based cell lysis using radioimmunoprecipitation (RIPA) buffer (50 mM Tris HCl, 150 mM NaCl, 2 mM EDTA and 1% NP-40, pH 7.5), 1% phosphatase inhibitors (P0044, Sigma-Aldrich, Darmstadt, Germany) and 4% protease inhibitors (11873580001, Roche Applied Science, Penzberg, Germany). Protein quantification for all samples was determined with the Bradford Assay Kit (Bio-Rad, Hercules, California, USA).

30 µg of protein (diluted in distillated water (dH20) if necessary) was mixed with loading buffer (LB) (containing 5% β-mercaptoethanol and 5% bromophenol blue in Laemmli 4× with Tris-HCl, 8% sodium dodecyl sulfate (SDS) and 40% glycerol). Protein samples were denatured at 95oC, for 5 minutes and separated by molecular masses in a 12% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE). The separated proteins were transferred to a nitrocellulose membrane using iBlot 2 Dry Blotting System (Thermo Fisher Scientific, Darmstadt, Germany), according to the producer's directives, at 20 V for 1 minute, 23 V for 4 minutes and 25 V for 2 minutes. After, the membranes were stained with Ponceau S. reagent (P7170-1L, Sigma-Aldrich, St. Louis, Missouri, USA). Membranes were blocked at room temperature, for 1 hour with 5% bovine serum albumin (BSA) (12659-500MG, EMD Millipore, Burlington, Massachusetts, USA) or 5% low-fat dry milk in Tris-buffered saline 1x with 0.1% Tween 20 (P1379, Sigma-Aldrich, St. Louis, Missouri, USA) (TBS-T 0.1%), accordingly on the dilution of the primary antibodies and manufacturer's instructions.

Primary antibodies used were anti-DRP1 (1:1000, DLP1 611113, BD Biosciences, Franklin Lakes, New Jersey, USA), anti-ERK1/2 (1:1000, P44/42 MAPK (ERK1/2), 9102S, Cell Signaling Technology , Danvers, Massachusetts, USA), anti-phospho-ERK1/2 (1:1000, p-P44/42 MAPK (T20214204), 9101S, Cell Signaling Technology, Danvers, Massachusetts, USA). Membranes were incubated with the primary antibodies at 4º C, overnight. Anti-α-tubulin (1:8000, T6074, Sigma-Aldrich, Darmstadt, Germany) was used as loading control. The membranes were incubated with anti-tubulin at 4º C, for an hour. Peroxidase labelled secondary antibodies were used depending on the host animal species in which the primary

antibody was produced (1:2000 GE Healthcare, Munich Germany or Santa Cruz, Heidelberg, Germany), all the secondary antibodies were diluted in 5% low-fat milk. The incubation period for the secondary antibodies was 1 hour at room temperature. Between each incubation, membranes were washed in TBS-T 0.1%. Enhanced chemiluminescence (ECL) with a 1:1 mix of Enhanced Luminol Reagent and the Oxiding Reagent (PerkinElmer, Whaltham Massachusetts, USA), and X-ray films (Amersham Hyperfilm ECL, GE Healthcare, Munich, Germany) were used for protein detection. Two protocols with different harshness were applied with the intuit to remove the previous primary and secondary antibodies. For the mild stripping, membranes remained in a solution composed by 0.2 M glycine, 1% of SDS and 10% of Tween 20, with a pH of 2.2. For the harsh stripping the membranes stayed in a buffer comprising 20% SDS 10%, 12.5% Tris-HCl 0.5M pH 6.8 and 0.8% β-mercaptoethanol, in the fume hood, at 50º C, for 45 minutes. The quantification of protein expression was obtained using the Bio-Rad Quantity One 1-D Analysis 4.6.9 Software (Bio-Rad, Hercules, California, USA).

Table 2 List of primary antibodies used for Western Blot

Primary Antibody	Reference	Host	Diluter	Dilution	Incubation period
Anti-DRP1	DLP1 611113, BD Biosciences	Mouse	BSA	1:1000	Overnight
Anti-ERK1/2	P44/42 MAPK (ERK1/2), 9102S, Cell Signaling Technology	Rabbit	Milk	1:1000	Overnight
Anti-phospho-ERK1/2	p-P44/42 MAPK (T20214204), 9101S, Cell Signaling Technology	Rabbit	BSA	1:1000	Overnight
Anti α-tubulin	T6074, Sigma-Aldrich	Mouse	Milk	1:8000	1 hour

3.7 mRNA Expression

For RNA expression, cells were plated in 6-well plates with a density of $1x10^5$, $1.5x10^5$ and $2x10^5$ cells/well for TPC1, XTC-1 and 8505C, and C643, respectively. 24 after seeding, cells were incubated in the conditions described in Table 2, except for Mdivi-1 25 and 50 μM and of Dabrafenib 15 μM, for 72h. At the end, cells were lysed with TripleX tractor reagent (GRiSP Research Solutions, Porto, Portugal) and RNA was extracted accordingly to the manufacturer's instructions. Briefly, media was removed and 1 mL of TripleXtractor reagent was added to the top of the cells, allowed to lyse cells for 1 minute and collected to an RNAse/DNase free tube. After addition of 200 μL of chloroform, it was carried out one centrifugation at 12,000 x g for 15 minutes. After, the aqueous (upper phase containing the RNA) was collected to a new tube and RNA was recovered by isopropanol precipitation. RNA concentration was determined using the NanoDrop ND-1000 Spectrophometer (Nanodrop Technologies, Inc., DE, USA).

DNase I (ThermoScientific, USA) was used to eliminate contaminating DNA from RNA prior to qRT-PCR. Briefly, 1 mg of total RNA was incubated with 10X reaction buffer with MgCl2, 1U of DNase I and water to a final volume of 10 mL for 30 minutes at 37º C. Later, 1 mL of 50mM EDTA was added and incubated for 10 minutes at 65º C.

Complementary deoxyribonucleic acid (cDNA) synthesis was performed using the previous mixture where DNA was removed. 1 mL of Random Hexamer Primers was added and incubated for 5 minutes at 65º C and immediately chilled on ice. Then, 8 μL of the following mixture were added: 4 μL of 5X Reaction Buffer, 20 U RiboLock RNase Inhibitor, 2 μL of dNTP Mix (10 mM) and 200 U of RevertAid Reverse Transcriptase (all from Thermo Scientific, USA). The reaction was incubated at 25º C for 10 minutes, followed by 42º C for 60 minutes and terminated at 70º C for 10 minutes. The reaction was performed on Bio-Rad MyCycleTM thermal cycle (BIO RAD, CA, USA). A negative control (-RT; water) was included to later check if there was contamination during cDNA synthesis.

Real-time quantitative polymerase chain reaction (RQ-PCR) was performed to evaluate the relative mRNA expression at the different treatment conditions of NIS, TSHr and

OCT4 using TBP as housekeeping gene. 100 ng of cDNA was amplified using 0.5 µL of PrimeTime® qPCR Assays for each gene of interest (IDT, USA), 5 µL of TaqMan™ Universal PCR Master Mix (ThermoScientific, USA) and water in a final volume of 10 µL. The mixture was incubated at 95º C for 10 minutes once, followed by 40 cycles of 95º C for 15 seconds and 60º C for 1 minute in the QuantStudio 5 Real-Time PCR System (ThermoScientific, USA). Triplicates were performed for each condition. -RT control, as well as a No Template Control (NTC: RT-qPCR control for contamination) were performed to check for contamination.

3.8 Statistical Analysis

For the immunochemistry data, continuous variables were summarized by mean and standard deviation and compared using Student's t-test or by median and minimum-maximum and compared using Wilcoxon's test. Shapiro-Wilk test was used to assess the normality of continuous variables. Categorical variables were summarized by number of cases and percentage and compared using Chi-square test or Fisher's exact test, as applicable. All analyses were conducted considering the complete cases for the variables analyzed. To assess the strength of the relationship between DRP1 and S616-p-DRP1 positive cases and other categorical variables, the odds ratio (OR) and respective 95% confidence intervals (CIs) were calculated. The Kaplan-Meier survival curves were plotted with the log-rank test statistics. The hazard ratio (HR) was then estimated by Cox proportional hazards regression. Kendal's correlation coefficient was calculated for the total DRP1 score and S616-p-DRP1 score. All statistical analyses were conducted using R Statistical Software (version 3.6.1) and the statistical significance level was set at 5%.

The data for the mechanistic cell studies were analyzed by one-way ANOVA followed by Turkey test (to correct for multiple comparisons) in GraphPadPrism 6.0 (GraphPad Software, Inc., La Jolia, California, USA). The data are presented as mean ± standard deviation (SD). A *P* value equal or superior to 0.05 was considered as statistically non-significant. A *P* value

between 0.01 and 0.05 was considered statistically significant, between 0.005 and 0.01 was considered very significant and between 0.001 and 0.005 was considered extremely significant.

4. Dynamin-related protein 1 expression in a large series of follicular cell derived thyroid carcinoma

This chapter appears as an article published in Endocrinology under the title “S616-p-DRP1 associates with locally invasive behavior of follicular cell-derived thyroid cancer” (Lima AR *et al*. S616-p-DRP1 associates with locally invasive behavior of follicular cell-derived thyroid cancer”. Endocrine. 2020 Nov 20. doi:10.1007/s12020-020-02546-4. Online ahead of print.).

S616-p-DRP1 associates with locally invasive behavior of follicular cell-derived thyroid cancer

Purpose: Dynamin-related protein 1 (DRP1), a mitochondrial fission protein, and its active form phosphorylated at Serine 616 (S616-p-DRP1) have been increasingly linked with tumorigenesis and invasion in various tumor models, including oncocytic thyroid cancer (TC). In this study, the expression of DRP1 and S616-p-DRP1 and its relationship with patients’ clinicopathological characteristics, tumor genetic profiles and clinical outcomes were assessed in a large series of follicular cell-derived TC (FCDTC).

Methods: Retrospective biomarker study characterizing the clinicopathological and immunochemistry DRP1 and S616-p-DRP1 expression of a series of 259 patients with FCDTC followed in two University Hospitals.

Results: DRP1 expression was positive in 65.3% (169/259) of the cases, while the expression of the S616-p-DRP1 was positive in only 17.3% (17/98). DRP1-positive expression was significantly associated with differentiated tumors (67.7% versus 48.0%; *P* = 0.049), non-encapsulated tumors (73.8% versus 57.4%; *P* = 0.011) and thyroid capsule invasion (73.4% versus 57.5%; *P* = 0.013). S616-p-DRP1-positive expression was significantly associated with tumor infiltratrive margins (88.9% versus 11.1%; P=0.033), thyroid capsule invasion (29.8% versus 3.1%; *P* = 0.043), lymph node metastases (23.3% versus 8.1%; *P* = 0.012) and higher

mean cumulative radioiodine dosage [317.4 ± 265.0 mCi versus 202.5 ± 217.7 mCi; *P* = 0.038]. S616-p-DRP1 expression was negatively associated with oncocytic phenotype (0.0% versus 26.2%; *P* = 0.028).

Conclusion: S616-p-DRP1 is a better candidate than DRP1 to identify tumors with locally invasive behavior. Prospective studies should be pursued to assess S616-p-DRP1 role as a molecular marker of malignancy in TC and in patients' risk assessment.

Introduction

Thyroid cancer (TC) is the most common endocrine malignancy, with a worldwide 5-year prevalence among all cancers of 4.6% by 2018 (https://gco.iarc.fr/today/online-analysis-pie, accessed July 12, 2020), ranking 9th place in the list of more common malignancies (1) TC presents a 3:1 higher incidence in women and includes diseases with remarkably different features varying from indolent localized papillary carcinoma to lethal anaplastic carcinoma (1, 2). It has been argued that the prognosis of TC depends more on the interplay between clinical and biological factors, including age, size, gender, histopathological features, and genetic factors, than from genetic factors alone (3–6). Both the International Union Against Cancer/American Joint Committee on Cancer staging system, which combines age and Tumor, Node, Metastases (TNM) staging to assess the risk of death due to TC, and clinico-pathologic features are accepted as prognostic indicators in TC. Less consensus exists about the role of genetic or molecular markers as individual prognosis measure. Amongst these, mutations in the telomerase reverse transcriptase (TERT) promoter and in TP53 have been retrospectively associated with a worse clinical outcome, but still require a prospective validation (7–9). B-Raf proto-oncogene (BRAF) and rat sarcoma viral oncogene (RAS) may also have a prognosis value under some circumstances, not yet fully clarified [3, 5].

Recently, our group reported an overall increase in the levels of “mitochondria-shaping” proteins in TC, suggesting a role for abnormal mitochondrial biogenesis and dynamics in thyroid cell transformation (10). From those, dynamin related protein 1 (DRP1)—the major player in mitochondrial fission—was the most highly expressed in TC (10). DRP1 is the largest member of the dynamin family of guanosine triphosphatase proteinases known to constrict membranes (11-14). It is mainly a cytosolic protein, but it translocates to mitochondria to promote mitochondrial fission after undergoing extensive posttranslational modifications altering its localization and affinity for oligomerization (12, 13, 15). DRP1 oligomerizes into spirals around the mitochondrial outer membrane, constricting the organelle through guanosine triphosphatase protein hydrolysis to promote mitochondrial fragmentation (13, 15, 16). Mitochondria division is needed in different and sometimes opposing processes,

such as apoptosis and cell cycle progression, mitosis, as well as in mitophagy (17, 18). This is achieved, at least partially, through the phosphorylation of DRP1 at serine residue 616—S6161-p-DRP1 (18, 19). The increased or enhanced activation of DRP1 has been associated with malignant phenotype in various epithelial and endocrine tumors (20–34). DRP1-based changes in mitochondrial dynamics have been associated with cell migration and invasion in TC, breast cancer, lung cancer and glioblastoma (10, 21, 23, 26). In TC, overexpression of DRP1 was also found to be associated with oncocytic tumors and, within these, with carcinoma (10). Interestingly, a higher expression of S616-p-DRP1 has been reported in *BRAF*^{V600E} mutated melanoma, and in ERK2- activated pancreatic cancer, with mechanistic work supporting the importance of this phosphorylation in tumorigenesis (29, 33–35). Mitogen-activated protein kinase (MAPK) has been identified as a key signaling pathway involved in DRP1 activation, with ERK1/ERK2 directly phosphorylating DRP1 (29, 33). When MAPK was inhibited, S616-p-DRP1, but not total DRP1, was reduced, supporting the translational importance of assessing this active form as opposed to total DRP1 in tumor samples [34]. Although most published studies reported on DRP1 expression, recent research has focused on S616-p-DRP1 assessment (20, 23, 29, 34).

Few studies have examined the significance of DRP1 overexpression in TC cells, and in the oncocytic variants in particular (10). It would be clinically relevant to assess if DRP1—or its activated form S616-p-DRP1—could have a role as a prognosis factor in the risk assessment of patients with DTC, an unmet medical need in those 5–10% of TC cases which will potentially have a poor outcome.

The aim of the present study was to assess the expression of DRP1 by immunohistochemistry (IHC) in a large series of patients with FCDTC, including oncocytic variants, which according with the new classification includes Hürthle cell carcinomas (HCC) (36), and to evaluate the relationship between its expression and the patients' clinicopathological characteristics, genetic or molecular profiles of the tumor, and clinical outcomes. We have also assessed the expression of S616-p-DRP1 in a sub-sample of our series and derived the same analyses described for DRP1.

Material and methods

Tumor samples

Our study included a series of 259 formalin-fixed, paraffin-embedded human tissue samples from FCDTC collected from the biobank of the Institute of Molecular Pathology and Immunology of the University of Porto (Ipatimup). The material used in this study originated from patients followed in two University Hospitals in Portugal - *Centro Hospitalar São João* (CHSJ) and *Centro Hospitalar e Universitário de Coimbra* and from the clinical databases of these hospitals. This work was approved by the local Ethics Committee for Health (CES), and all the procedures described in this study followed the national legal requirements and the Helsinki declaration.

The demographic and clinicopathological data of the patients were retrospectively collected from the histopathological reports and clinical databases. The histology of all tumor samples was reviewed independently by two pathologists (ER and MSS), and the thyroid tumor classification was performed according to the WHO criteria (2). Patients were stratified by clinicopathological characteristics in the following categories: gender, age (≥45 years or <45 years), histological diagnosis, TNM (37) stage, tumor size, tumor capsule invasion, vascular invasion, thyroid capsule invasion, extrathyroidal invasion, multifocality, presence of lymph node and distant metastases, presence of $BRAF^{V600E}$ mutations, presence of *TERT* promoter mutations (-124G>A and -146G>A), cumulative dosage of radioiodine treatment, persistence of the disease at the end of follow-up, disease-specific mortality, and overall mortality. «Aggressive variants» were defined as all cases of solid/trabecular, diffuse sclerosing, tall cell or columnar cell PTC, as well as all cases of PDTC. When comparing PTC with FTC and FVPTC, «aggressive variants» were excluded. We performed an analysis for the whole sample and a subanalysis for the major histotypes. Considering that FTC and FVPTC share morphological (follicular pattern) and molecular features (high proportion of *RAS* mutations), we also considered a subgroup encompassing these two types of tumors (115).

Immunohistochemical analysis

Immunohistochemistry (IHC) was performed in 3-µm formalin-fixed, paraffin-embedded sections. Sections were deparaffinized and rehydrated in a series of decreasing concentrations of ethanol solutions. Deparaffinized sections were subject to heat-induced antigen retrieval in 1 mM pH 9.0 ethylenediaminetetraacetic acid buffer (EDTA) (LabVision Corporation, Fremont, CA, USA). Endogenous peroxidase activity was blocked with UltraVision Hydrogen Peroxide Block and non-specific bind was blocked using UltraVision Block reagent from UltraVision Quanto Detection System HRP DAB (Thermo Scientific/Lab Vision, Fremont, USA) for 10 minutes. The sections were then incubated in a humidified chamber, according to the manufacturer's specifications, with the following primary antibodies: mouse monoclonal antibody for DRP1 (1:100) ref. 611112 (BD Biosciences), rabbit polyclonal antibody for S616-p-DRP1 (1:500) ref. 3455, from Cell Signaling. The sections were then washed and stained by using the UltraVision Quanto Detection System HRP DAB (Thermo Scientific/Lab Vision, Fremont, USA). All sections were counterstained with Mayer's hematoxylin. Positive controls from previously tested kidney samples were used in every run. To assess the specificity of the immunostaining, tumor sections not incubated with the primary antibody were used as negative controls. Whenever present, scattered macrophages and muscular tissue were considered as internal positive controls for both proteins. A second internal and positive control for each tumor sample was the expression of both proteins in normal adjacent thyroid tissue.

Evaluation of immunohistochemical staining

Immunostaining was semi-quantitatively evaluated by three observers (ARL, LS, and VM for DRP1 and SC, CT, and VM for S616-p-DRP1) without the knowledge of any clinical information of the cases. The expression of DRP1 in tumor tissue was evaluated according to an immune-reactive staining score (IRS) adapted from other studies (39-41) Immunohistochemical positivity was defined as cytoplasmic staining for DRP1 and S616-p-

DRP1, and immunostaining scores were based on the intensity and the extension of tumor cells immunostaining, as described in Table 2. A total IRS was then obtained by multiplying the intensity (I) and extension (E) scores, i.e., IRS = I x E, ranging from 0 to 12. Positive expression or overexpression of DRP1 and S616-p-DRP1 in TC sections was defined by an IRS of 6 or higher. This positivity criterion was based on previous observations that the immunostaining of DRP1 in normal adjacent thyroid tissue was usually weaker than in neoplastic tissue with an IRS score of 4 or lower.

Statistical analysis

Continuous variables were summarized by mean and standard deviation and compared using Student's t-test or by median and minimum-maximum and compared using Wilcoxon's test. Shapiro-Wilk test was used to assess the normality of continuous variables. Categorical variables were summarized by number of cases and percentage and compared using Chi-square test or Fisher's exact test, as applicable. All analyses were conducted considering the complete cases for the variables analyzed. To assess the strength of the relationship between DRP1 and S616-p-DRP1 positive cases and other categorical variables, the odds ratio (OR) and respective 95% confidence intervals (CIs) were calculated. The Kaplan-Meier survival curves were plotted with the log-rank test statistics. The hazard ratio (HR) was then estimated by Cox proportional hazards regression. Kendal's correlation coefficient was calculated for the total DRP1 score and S616-p-DRP1 score. All statistical analyses were conducted using R Statistical Software (version 3.6.1) and the statistical significance level was set at 5%.

Table 1

Scoring system for the immunostaining of DRP1 and S616-p-DRP1 in thyroid cancer sections

Intensity (I)		**Extension (E)**	
Staining strength	Score	% of stained tumor cells	Score
Absent	0	≤10	0
Weak	1	11-25	1
Moderate	2	26-50	2
Strong	3	51-75	3
		>75	4

DRP1, dynamin-related protein 1, S616-p-DRP1, Serine 616-phosphorylated DRP1

Results

Patient characteristics and clinicopathological variables

Table 2 summarizes the patient and tumor characteristics. This study included samples from patients aged 11 to 83 years, 80.3% of whom were females. The TC cases included 162 cases of PTC (8 oncocytic and 154 non-oncocytic), 63 cases of follicular variant of PTC (FVPTC, 10 oncocytic and 53 non-oncocytic), 25 cases of FTC (11 oncocytic and 14 non-oncocytic) and 9 cases of PDTC (2 oncocytic and 7 non-oncocytic) (Table 2 and Fig.1). For simplicity, we have included HCC - previously described as variant of FTC -, within the FTC group. Most tumors were classified as stage I (47.1%). From the tumors for which data was available, 50.2% were capsulated, of which 80.0% presented capsule invasion. Vascular invasion was present in 35.9%, 49.1% had thyroid capsule invasion and 37.0% had extrathyroidal extension. Lymph node involvement was present in 36.2% of the patients, and 13.6% had distant metastases during follow-up. 40.9% of the tumors assessed for mutations presented the *BRAF*^{V600E} mutation, with 51.4% in PTC cases, and 12.3% presented *TERT* promoter mutation. The majority of patients (84.6%) were treated with radioiodine, with a mean cumulative dose of 195.1 ± 235.1 mCi. The median follow-up time for all patients was 7.5 (3.9

- 10.9) years. At the time of the last follow-up, 28.8% of patients had persistent disease and 6.6% patients had died, 55.9% of them due to TC.

Table 2

Characteristics of patients and tumors

Clinicopathological characteristics	DRP1 IRS score			S616-p-DRP1 IRS score		
	Total sample (N = 259, 100%)	Negative[a] (n = 90, 34.7%)	Positive[b] (n = 169, 65.3%)	Total sample (N = 98)	Negative[a] (n = 81, 82.7%)	Positive[b] (n = 17, 17.3%)
Age at diagnosis, years						
≥45, n (%)	135 (52.1)	46 (34.1)	89 (65.9)	59	53 (89.8)	6 (10.2)
<45, n (%)	124 (47.9)	44 (35.5)	80 (64.5)	39	28 (71.8)	11 (28.2)
Mean ± SD	45.5 ± 16.3	45.9 ± 17.8	45.3 ± 15.5	48.1 ± 16.5	49.8 ± 16.1	39.9 ± 17.4
Gender, n (%)						
Female	208 (80.3)	69 (33.2)	139 (66.8)	73	61 (83.6)	12 (16.4)
Male	51 (19.7)	21 (41.2)	30 (58.8)	25	20 (80)	5 (20)
Histological diagnosis, n (%)						
PTC	162 (62.5)	47 (29)	115 (71.0)	66	58 (87.9)	8 (12.1)
FVPTC	63 (24.3)	29 (46)	34 (54.0)	13	11 (84.6)	2 (15.4)
FTC	25 (9.7)	9 (36)	16 (64.0)	13	13 (100)	0 (0)
PDTC	9 (3.5)	5 (55.6)	4 (44.4)	6	5 (83.3)	1 (16.7)
Oncocytic variants, n (%)[a]						
n	247	85 (34.4)	162 (65.6)	94	77 (81.9)	17 (18.1)
Yes	32 (13.0)	9 (28.1)	23 (71.9)	29	29 (100)	0 (0)
No	215 (87.0)	76 (35.3)	139 (64.7)	65	48 (73.8)	17 (26.2)
TNM stage, n (%)						
I	122 (47.1)	39 (32)	83 (68.0)	39	31 (79.5)	8 (20.5)
II	24 (9.3)	8 (33.3)	16 (66.7)	5	5 (100)	0 (0)
III	54 (20.8)	18 (33.3)	36 (66.7)	23	19 (82.6)	4 (17.4)
IV	59 (22.8)	25 (42.4)	34 (57.6)	31	26 (83.9)	5 (16.1)
Tumor size						
n	251	85 (33.9)	166 (66.1)	94	79 (84)	15 (16)
Mean ± SD, cm	27.2 ± 16.4	28.9 ± 18.1	25.8 ± 15.3	29.9 ± 17.8	31.3 ± 18.3	23.0 ± 7.3
≤4 cm	208 (82.9)	67 (32.2)	141 (67.8)	74	60 (81.1)	14 (18.9)
>4 cm	43 (17.1)	18 (41.9)	25 (58.1)	20	19 (95)	1 (5)

Encapsulated tumors, n (%)						
n	215	74 (34.4)	141 (65.6)	79	65 (82.3)	14 (17.7)
Yes	108(50.2)	46 (42.6)	62 (57.4)	41	38 (92.7)	3 (7.3)
No	107(49.8)	28 (26.2)	79 (73.8)	38	27 (71.1)	11 (28.9)
Invasion, n (%)						
Tumor capsule						
n	100	45 (45)	55 (55.0)	35	32 (91.4)	3 (8.6)
Yes	80 (80.0)	36 (45)	44 (55.0)	30	27 (90)	3 (10)
No	20 (20.0)	9 (45)	11 (55.0)	5	5 (100)	0 (0)
Vascular						
n	223	77 (34.5)	146 (65.5)	79	65 (82.3)	14 (17.7)
Present	80 (35.9)	28 (35)	52 (65.0)	37	30 (81.1)	7 (18.9)
Absent	143(64.1)	49 (34.3)	94 (65.7)	42	35 (83.3)	7 (16.7)
Thyroid capsule						
n	222	77 (34.7)	145 (65.3)	79	64 (81)	15 (19)
Present	109 (49.1)	29 (26.6)	80 (73.4)	47	33 (70.2)	14 (29.8)
Absent	113(50.9)	48 (42.5)	65 (57.5)	32	31 (96.9)	1 (3.1)
Extrathyroidal						
n	227	79 (34.8)	148 (65.2)	80	65 (81.2)	15 (18.8)
Present	84 (37.0)	23 (27.4)	61 (72.6)	38	27 (71.1)	11 (28.9)
Absent	143 (63.0)	56 (39.2)	87 (60.8)	42	38 (90.5)	4 (9.5)
Multifocality, n (%)						
n	228	79 (34.6)	149 (65.4)	80	65 (81.2)	15 (18.8)
Present	83 (36.4)	25 (30.1)	58 (69.9)	37	28 (75.7)	9 (24.3)
Absent	145(63.6)	54 (37.2)	91 (62.8)	43	37 (86)	6 (14)
Lymph node metastases, n (%)						
n	257	88 (34.2)	169 (65.8)	97	80 (82.5)	17 (17.5)
Present	93 (36.2)	34 (36.6)	59 (63.4)	60	46 (76.7)	14 (23.3)
Absent	164 (63.8)	54 (32.9)	110 (67.1)	37	34 (91.9)	3 (8.1)
Distant metastases, n (%)						
n	258	90 (34.9)	168 (65.1)	97	97 (89.7)	10 (10.3)
Present	35 (13.6)	16 (45.7)	19 (54.3)	16	14 (87.5)	2 (12.5)
Absent	223 (86.4)	74 (33.2)	149 (66.8)	81	73 (90.1)	8 (9.9)
Molecular diagnosis, n (%)						

***BRAF*^{V600E} mutation**						
n	215	75 (34.9)	140 (65.1)	76	59 (77.6)	17 (22.4)
Positive	88 (40.9)	27 (30.7)	61 (69.3)	36	30 (83.3)	6 (16.7)
Negative	127 (59.1)	48 (37.8)	79 (62.2)	40	29 (72.5)	11 (27.5)
***TERT* promotor mutation**						
n	187	68 (36.4)	119 (63.6)	66	50 (75.8)	16 (24.2)
Positive	23 (12.3)	11 (47.8)	12 (52.2)	9	8 (88.9)	1 (11.1)
Negative	164 (87.7)	57 (34.8)	107 (65.2)	57	42 (73.7)	15 (26.3)
Radioiodine treatment, n (%)						
Yes	219 (84.6)	73 (33.3)	146 (66.7)	83	66 (79.5)	17 (20.5)
No	40 (15.4)	17 (42.5)	23 (57.5)	15	15 (100)	0 (0)
No. of doses, n (%)						
n	219	73 (33.3)	146 (66.7)	82	65 (79.3)	17 (20.7)
1	140 (63.9)	39 (27.9)	101 (72.1)	44	36 (81.8)	8 (18.2)
≥2	79 (36.1)	34 (43)	45 (57.0)	38	29 (76.3)	9 (23.7)
Cumulative iodine dosage,						
n	241	84 (34.9)	157 (65.1)	91	74 (81.3)	17 (18.7)
mean ± SD, mCi	195.1 ± 235.1	230.0 ± 285.5	176.5 ± 201.6	223.9 ± 230.1	202.5 ± 217.7	317.4 ± 265.0
Disease-free status at the end of follow-up, n (%)						
n	236	78 (33.1)	158 (66.9)	90	73 (81.1)	17 (18.9)
Yes	168 (71.2)	50 (29.8)	118 (70.2)	57	47 (82.5)	10 (17.5)
No	68 (28.8)	28 (41.2)	40 (58.8)	33	26 (78.8)	7 (21.2)
Follow-up time, mean ± SD, years	8.6 ± 6.4	9.4 ± 6.5	8.2 ± 6.4	8.1 ± 6.0	6.6 ± 6.0	8.8 ± 5.1
Overall mortality	17 (6.6)	9 (52.9)	8 (47.1)	9	8 (88.9)	1 (11.1)
Disease-related mortality	9 (3.5)	3 (33.3)	6 (66.7)	5	5 (100)	0 (0)

BRAF, B-Raf proto-oncogene; DRP1, dynamin-related protein 1; FTC, follicular thyroid carcinoma; FVPTC, follicular variant of papillary thyroid carcinoma; IRS, immune-reactive staining; mCi, millicurie; n, number of cases; PDTC, poorly differentiated thyroid carcinoma; Pos, positive; PTC, papillary thyroid

carcinoma; SD, standard deviation; S616-p-DRP1, Serine 616-phosphorylated DRP1; TERT, telomerase reverse transcriptase; TNM, tumor, node and metastasis

(a) negative DRP1 expression was defined as IRS <6

(b) positive DRP1 expression was defined as IRS ≥6

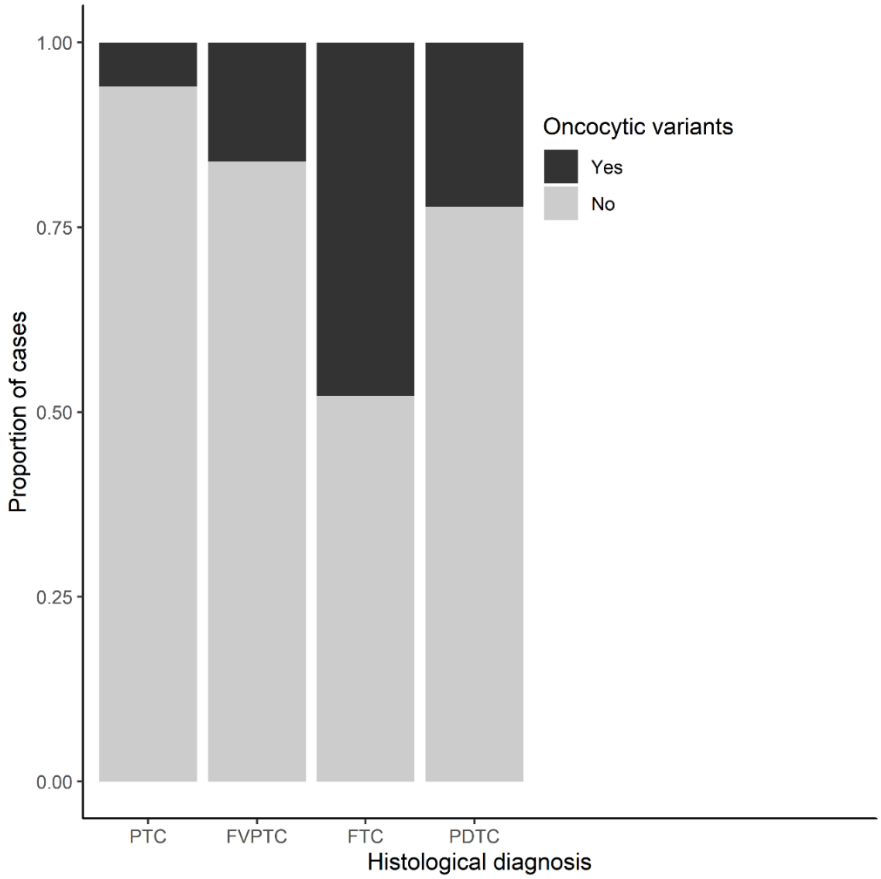
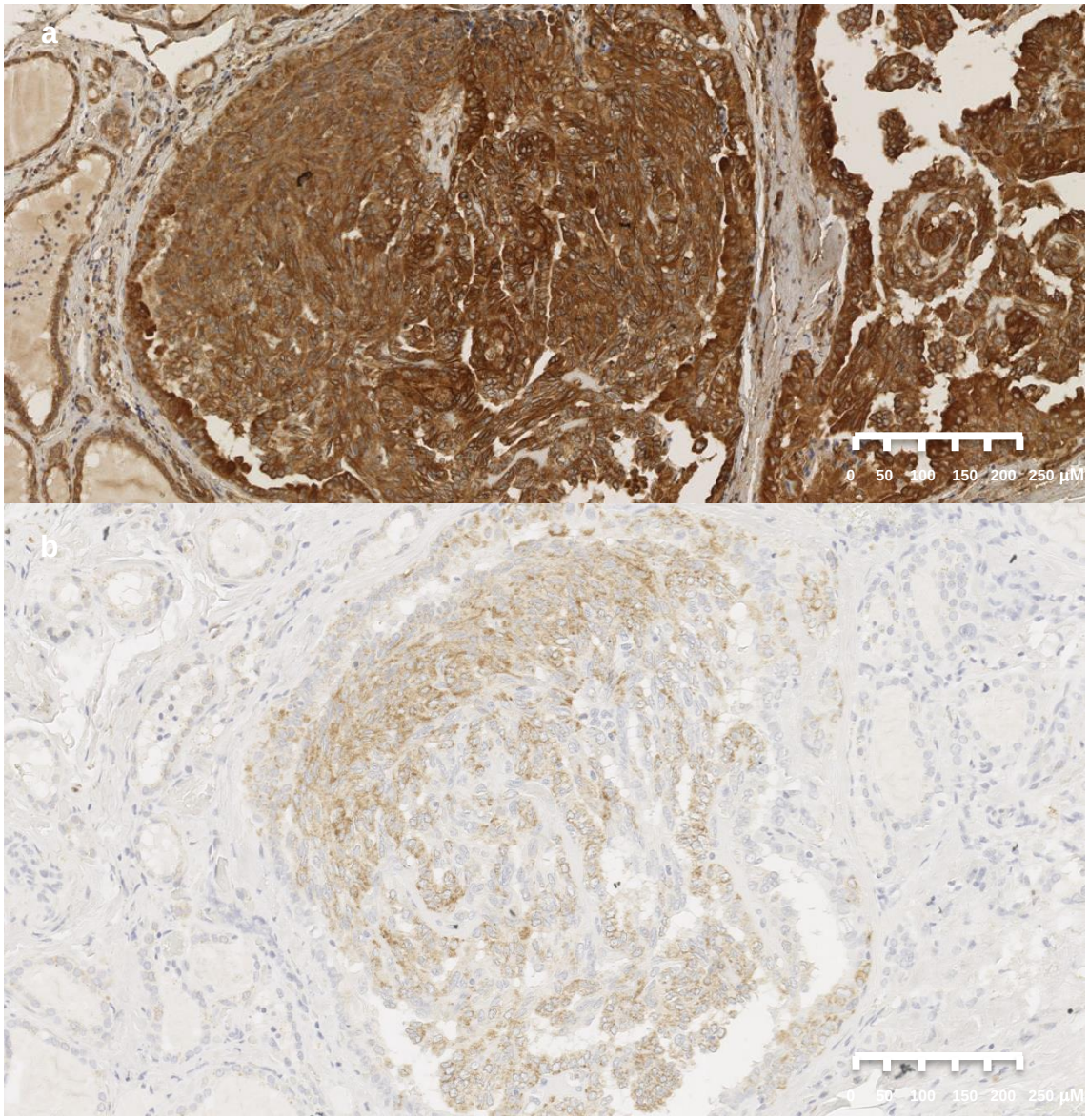


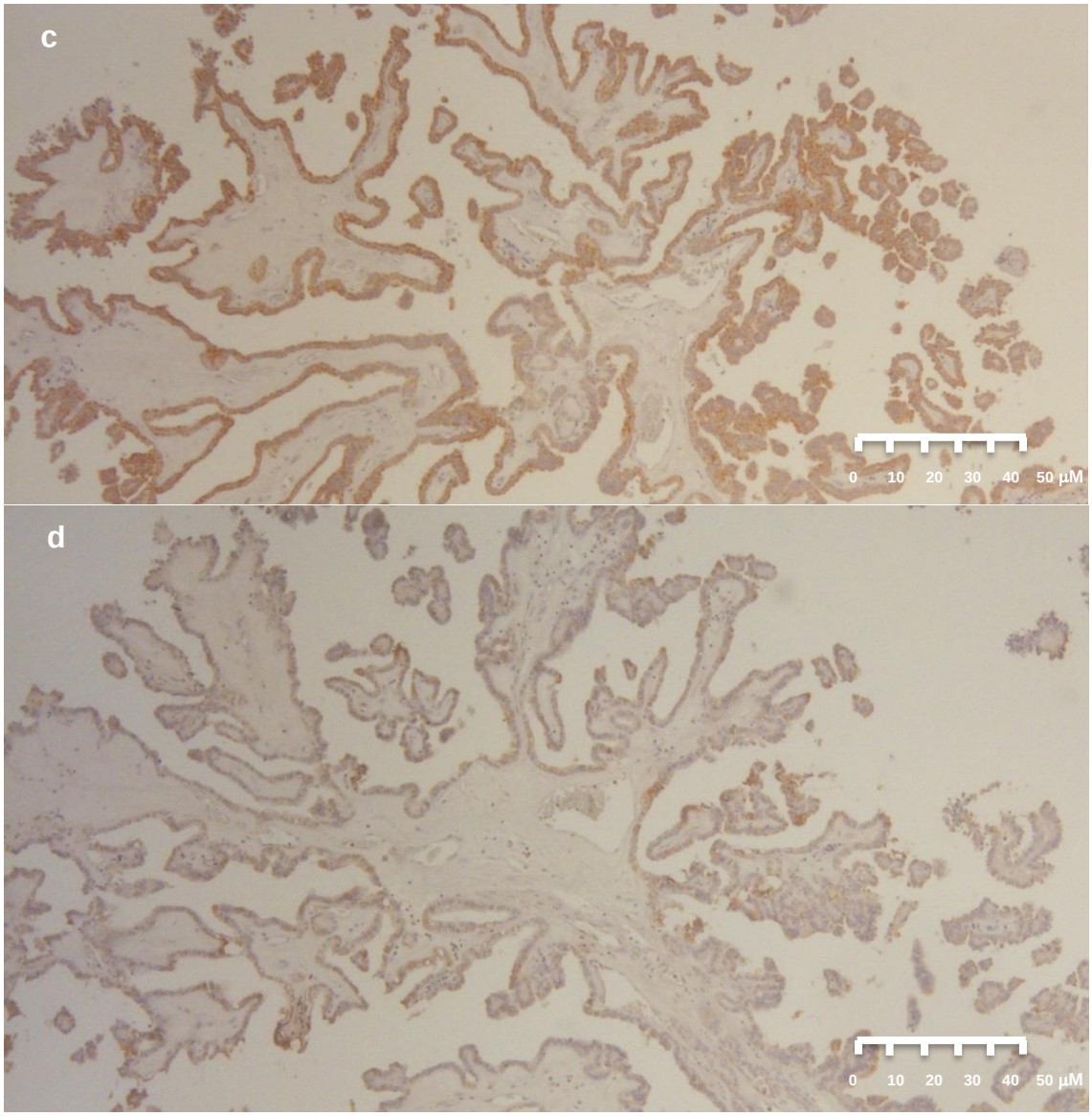
Fig. 1 Distribution of the histological type stratified by the presence or absence of oncocytic variants in the 259 TC cases. Black bars (yes) represent the reported proportion of oncocytic variant tumors in each histological type of TC. FTC, follicular thyroid carcinoma; FVPTC, follicular variant of papillary thyroid carcinoma; PDTC, poorly differentiated thyroid carcinoma; PTC, papillary thyroid carcinoma; TC, thyroid cancer

DRP1 expression in FCDTC and correlation with S616-p-DRP1

DRP1 expression was positive in 65.3% (169/259) of TC cases in this study (Table 2). Out of the 98 TC cases tested for S616-p-DRP1, 17.3% (17/98) were positive for the expression of the active form of DRP1. Both DRP1 and S616-p-DRP1 antibodies showed cytoplasmic staining, with no nuclear staining (Figs. 2a, b). Critically, unlike DRP1, p-616-DRP1 did not stain normal thyroid tissue.

No significant correlation was found between the expression of total DRP1 and S616-p-DRP1 (Kendall's Tau correlation coefficient = 0.063, *P* = 0.451) (Figs. 2c, d). Despite this, the expression of both DRP1and S616-p-DRP1 was positively associated with more differentiated histologies and locally invasive traits, as described below.




c
0 10 20 30 40 50 µM
d
0 10 20 30 40 50 µM

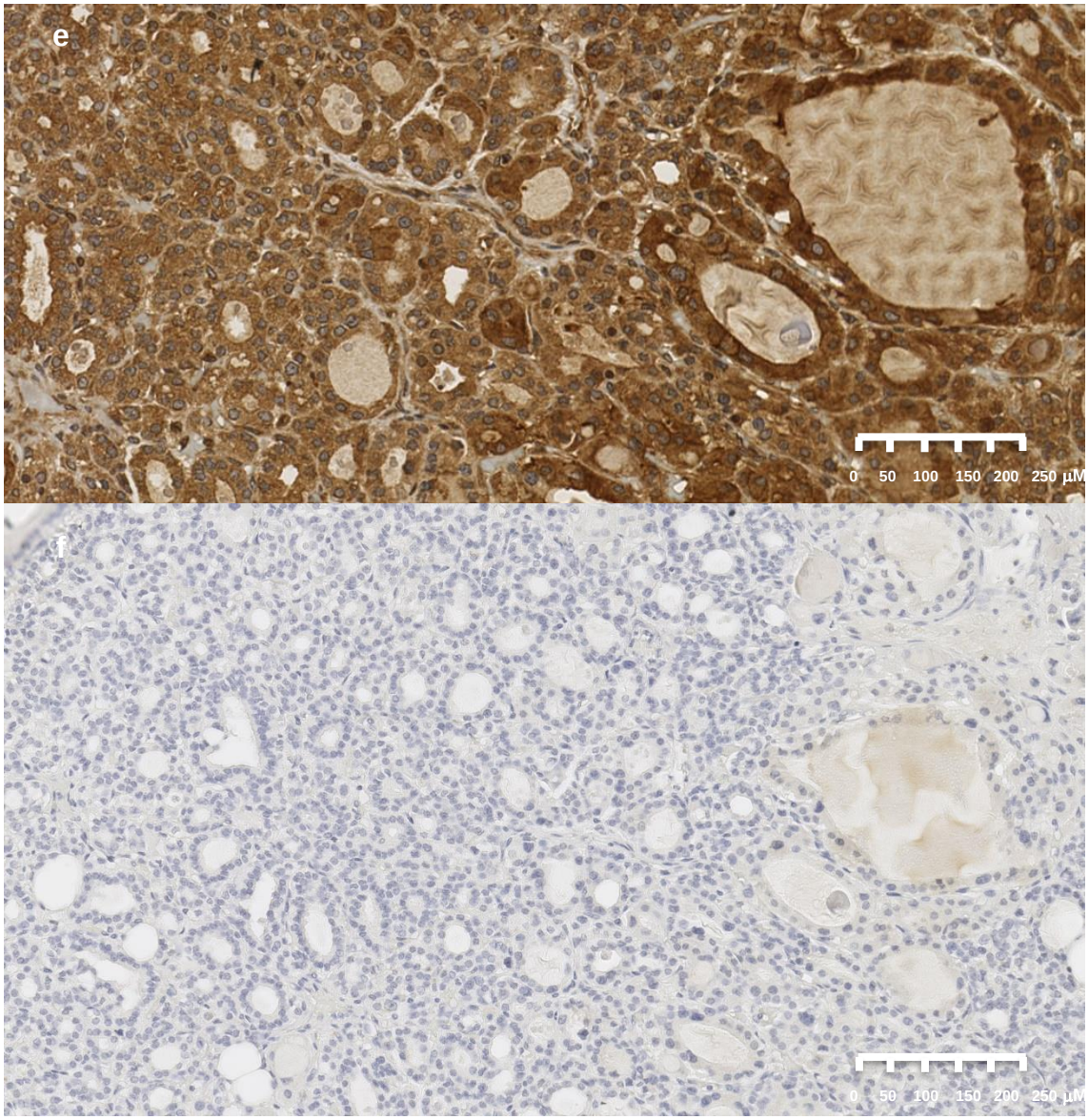


Fig. 2 Representative photomicrographs of the immunohistochemical analysis of DRP1 and S616-p-DRP1 staining in thyroid cancers. a Typical DRP1 expression is cytoplasmic without nuclear expression in a cPTC case with IRS score 9, ×20 magnification; b Typical S616- p-DRP1 expression is cytoplasmic without nuclear expression in a cPTC case with IRS score 2, ×20 magnification; c DRP1 expression pattern of a PTC case with IRS score 2, ×4 magnification; d S616-pDRP1 expression pattern of the same PTC case as (c) with IRS score 12, ×4 magnification. e Oncocytic cells with high expression of DRP1 as assessed by an IRS score = 12, ×20 magnification; f Oncocytic cells with low expression of S616-p-DRP1 as assessed by an IRS score = 0, ×20 magnification. cPTC classical papillary thyroid carcinoma, DRP1 dynamin-related protein 1, IRS immune-reactive staining, PTC papillary thyroid carcinoma, S616-p-DRP1 serine 616-phosphorylated DRP1, TC thyroid cancer

DRP1 expression according to clinico-pathological characteristics

DRP1 was positive in 71.0%, 64.0% and 44.4% for PTC, FTC and PDTC, respectively. No differences in DRP1 expression were found between PTC and FTC. However, PTC presented a significantly higher proportion of DRP1-positive cases when compared with FVPTC and FTC combined (73.8% versus 56.9%; *P* = 0.007) (Fig. 3a). Similarly, the proportion of DRP1-positive cases was significantly higher in «non-aggressive» compared with «aggressive» variants (67.7% versus 48.0%; *P* = 0.049) (Fig. 3b). No associations were found between DRP1 expression and the presence of oncocytic variants (71.9% versus 64.7%; *P* =0.422).

Non-encapsulated tumors had a significantly higher rate of DRP1-positivity in comparison with encapsulated tumors (73.8% versus 57.4%; *P* = 0.011) (Fig. 3c). Tumors with thyroid capsule invasion had a significantly higher rate of DRP1-positivity (73.4% versus 57.5%; *P* = 0.013) (Fig. 3d).

Patients subjected to more than one radioiodine treatment harbored tumors with significantly lower DRP-1 expression (30.8% versus 69.2%; *P* = 0.022) (Fig. 4).

No significant association was found between DRP1 expression and gender, age, tumor stage, tumor size, vascular invasion, extrathyroidal invasion, multifocality, lymph node or distant metastases, *BRAF*^{V600E} mutation, *TERT* promoter mutation, cumulative radioiodine dose, disease status at the end of follow-up, disease-related mortality or overall mortality (Supplementary Tables 1, and Supplementary Figs. 1 and 2).

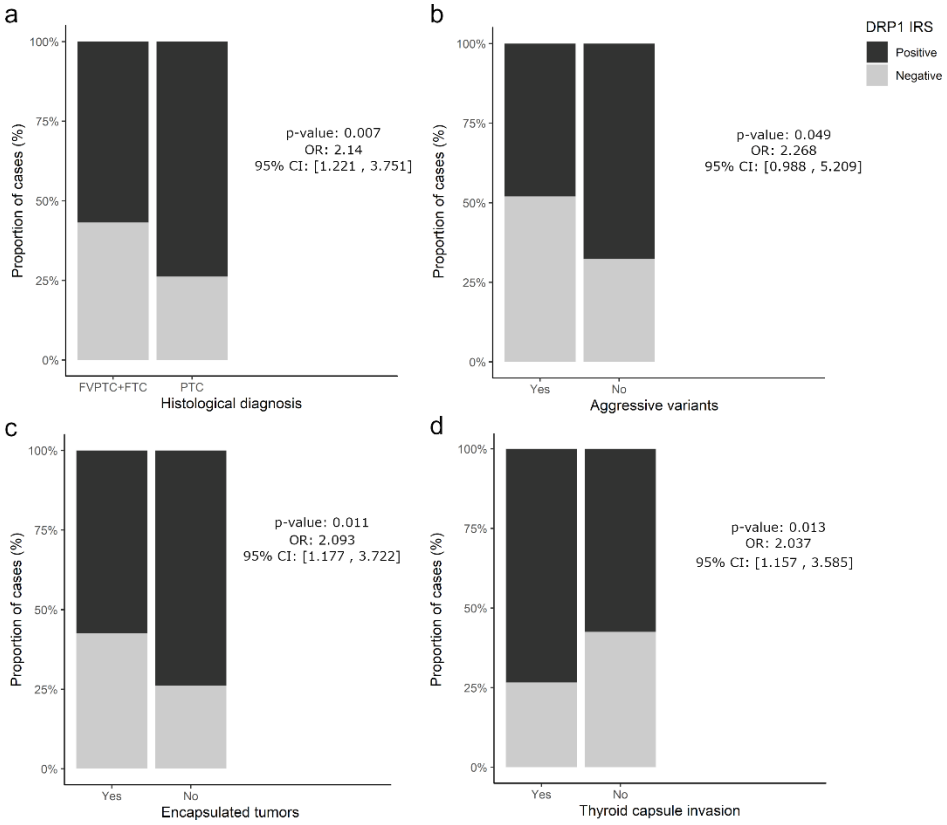


Fig. 3 Expression of DRP1 in (a) classic PTC cases vs. combined FVPTC and FTC cases; b more aggressive histologies - as defined in “Material and methods” section - vs. less aggressive histologies - differentiated TC (DTC) excluding “aggressive variants”; c encapsulated vs. non-encapsulated tumors; d tumors with vs. tumors without thyroid capsule invasion. Poor prognosis variants of PTC (e.g., solid/ trabecular, diffuse sclerosing, tall cell, or columnar cell) were excluded from analysis (a). Black bars represent positive DRP1 expression (defined as IRS ≥ 6) and gray bars represent negative DRP1 expression (defined as IRS < 6). CI confidence interval, DRP1 dynamin-related protein 1, FTC follicular thyroid carcinoma, FVPTC follicular variant of papillary thyroid carcinoma, IRS immune-reactive staining, OR odds ratio, PTC papillary thyroid carcinoma

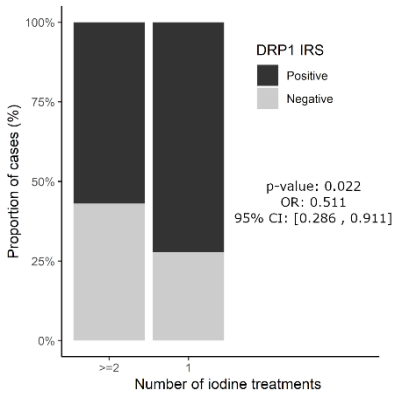


Fig. 4 Expression of DRP1 based on the number of radioiodine treatments. Black bars represent positive DRP1 expression (defined as IRS ≥6) and grey bars represent negative DRP1 expression (defined as IRS <6). CI, confidence interval; DRP1, dynamin-related protein 1; OR, odds ratio

S616-p-DRP1 expression according to clinicopathological characteristics

Patients who were less than 45 years presented a significantly higher S616-p-DRP1-positive expression rate versus those who were 45 years or more (28.2% versus 10.2 %; *P* = 0.013).

The rate of S616-p-DRP1 positivity was 12.1%, 0.0% and 16.7% for PTC, FTC and PDTC, respectively (Table 2). Surprisingly, and unlike what was seen for DRP1 (Fig. 2e), none of the oncocytic variant cases were positive for S616-p-DRP1 expression (0.0% versus 26.2%; *P* = 0.028) (Figs. 2f and 5a).

Tumors with infiltrative margins, or with infiltrative and expansive margins combined in the same tumor, presented a significantly higher proportion of S616-p-DRP1-positive cases when compared with tumors with expansive margins (88.9% versus 11.1%; *P* = 0.033) (Fig. 2c, d, showing high expression of S616-p-DRP1 and low expression of DRP1 in a PTC with infiltrative margins, and Fig. 5b). The same trend was observed for tumors with thyroid capsule invasion (29.8% versus 3.1%; *P* = 0.04) (Fig. 5c). There were also more S616-p-DRP1-positive cases in patients with lymph node metastases (23.3% versus 8.1%; *P* = 0.012) (Fig. 5d).

A statistically significant higher mean radioiodine cumulative dose was observed in patients who were positive for S616-p-DRP1 (317.4 ± 265.0 mCi vs. 202.5 ± 217.7 mCi; *P* = 0.038). Interestingly, all 5 cases of patients who died due to TC were negative for S616-p-DRP1, and 14/16 cases of patients who presented distant metastases were also negative for S616-p-DRP1 (Supplementary Table 2).

No significant association was found between S616-p-DRP1 expression and gender, tumor stage, tumor size, vascular invasion, extrathyroidal invasion, multifocality, distant metastases, *BRAF*^{V600E} mutation, *TERT* promoter mutation, cumulative radioiodine dose, disease status at the end of follow-up, disease-related mortality or overall mortality (Supplementary Table 1, and Supplementary Figs. 3 and 4).

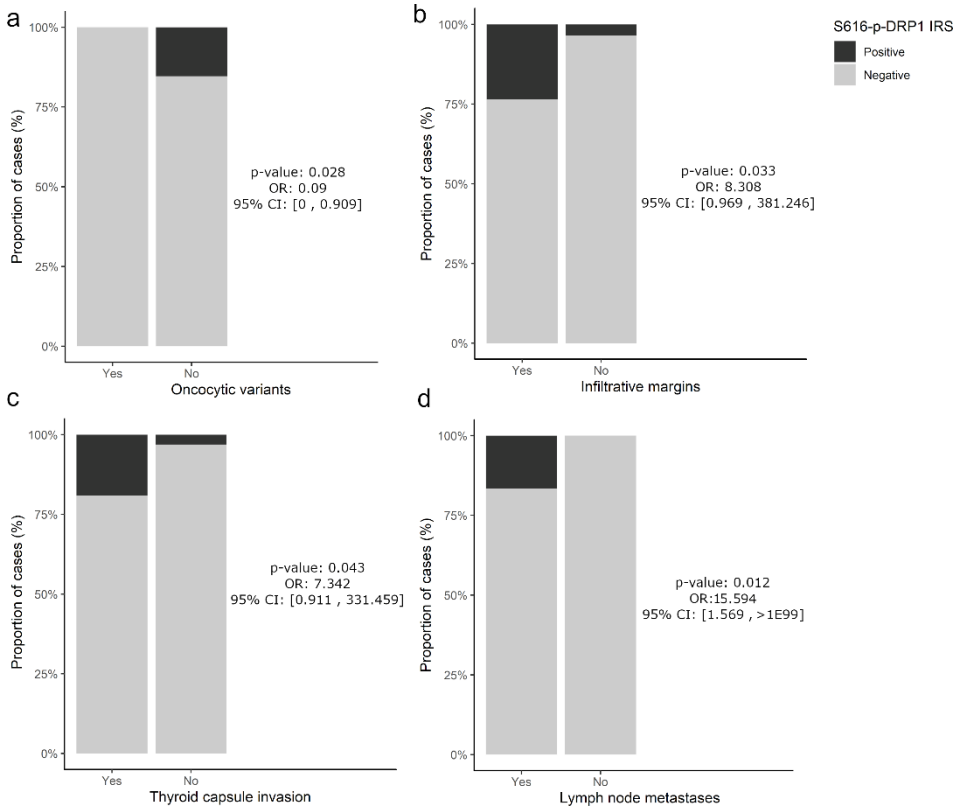


Fig. 5 Expression of S616-p-DRP1 in (a) TC cases by oncocytic vs. non-oncocytic carcinoma variants; b tumors with infiltrative margins vs. tumors with expansive margins; c tumors with vs. tumors without thyroid capsule invasion; d tumors with vs. tumors without lymph node metastases. Black bars represent positive DRP1 expression (defined as IRS ≥ 6) and gray bars represent negative DRP1 expression (defined as IRS < 6). CI confidence interval, IRS immune-reactive staining, OR odds ratio, S616-p-DRP1 serine 616- phosphorylated DRP1

Discussion

We studied the expression level of DRP1 and its active form, S616-p-DRP1, in a large series of FCDTC. Although there was no correlation between the expression of DRP1 and S616-p-DRP1, both were associated with locally invasive characteristics of the tumor. Aligned with reports in other tumor models (10, 21, 23, 42), the expression of DRP1 and S616-p-DRP1 was positively associated with locally invasive traits, as supported by a significantly higher number of positive tumors with thyroid capsule invasion. However, no significant association with poor prognosis factors was shown. Unlike DRP1, which showed positive in a large proportion of tumors (65.3%), S616-p-DRP1 was expressed in only 17.3% of those tested. This supports the correlative functional significance of S616-p-DRP1, as only a smaller fraction of DRP1 is relocated to the mitochondrial membrane as S616-p-DRP1 (43). The higher proportion of S616-p-DRP1-positive cases was significantly associated with infiltrative margins, thyroid capsule invasion, and lymph node invasion, in line with previous reports suggesting a role of S616-p-DRP1 in tumor invasiveness in breast and lung cancer (21, 23).

“Aggressive variants” of TC, which in our analysis also included PDTC, were associated with a significantly lower proportion of DRP1-positive expression, in comparison with DTC. It is tempting to hypothesize that tumors with lower DRP1 expression might reflect the phenotypic expression of less differentiated tumors of most “aggressive variants”. In agreement with this is the higher expression of S616-p-DRP1 in younger patients, which probably reflects the phenotype of a self-regulated replicative tumor with a higher level of differentiation. This would not only resonate with the better prognosis of this patients’ age group but also explain the apparent better response to the radioiodine therapy of these tumors, a concept that has been defended in other tumor models (20, 25).

Another point in favor of a link between higher DRP1 expression/activity and FCDTC differentiation is the significant association between lower expression of DRP1 and a higher number of radioiodine treatments. Interestingly, a statistically significant association between higher S616-p-DRP1 positive expression and a higher mean cumulative radioiodine dosage was observed. It is reasonable to admit that patients with locally invasive disease, including

lymph node metastases, may have been treated with higher radioiodine dosages, even if the number of radioiodine treatments has not significantly differed in the overall patient population tested. In this respect, the apparent contradictory results between radioiodine treatment intensity observed for total DRP1 and S616-p-DRP1 expression could be justified. We did not find any association between DRP1 or S616-p-DRP1 expression and distant metastases. Although the number of patients who presented distant metastases whose primary tumors were tested for S616-p-DRP1 was low, it is still noteworthy that 14 out of 16 were negative for S616-p-DRP1. Together with the fact that S616-p-DRP1 expression was associated with lymph node metastases, these data reinforce the idea that local and distant metastases have different molecular signatures in FCDTC, a concept that we discussed in a previous study of our group (44). On the other hand, it is also possible that the lower S616-p-DRP1 expression represents a phenotype of cells less metabolically adapted to higher oxygen concentrations, and thereby less prone to blood-borne metastization. Indeed, the association between the loss of DRP1 with impaired glycolytic flux and the loss of mitochondrial metabolic function has already been described (45).

In FCDTC, only a relatively small number of patients will eventually die from the disease. This observation turns it difficult to relate DRP1 expression with a long-term clinical outcome. Nevertheless, our results showing a trend towards lower S616-p-DRP1 positivity in patients who died from the disease seem to be in line with the lower number of DRP1-positive cases in less differentiated tumors.

One of the most puzzling results of our study was the fact that S616-p-DRP1-positive cases were significantly lower in the 28 oncocytic variants of TC. We would expect that, given the higher mitochondrial biogenesis and deficient mitophagy processes described in these variants (46–54), S616-p-DRP1 would be more highly expressed in these tumors (55). Our group has shown that DRP1 is overexpressed in oncocytic thyroid tumors, particularly HCC, suggesting that mitochondrial dynamics are dysregulated in Hürthle cells and that DRP1 might play a role in oncocytic tumorigenesis (10). DRP1 is kept in an equilibrium between cytosolic

and mitochondrial compartments (43, 56). Recent data suggest that mitochondrial DRP1 may account for 40–50% of the overall DRP1 cell population (43, 56).

Mitochondrial fission is a complex process, which is dependent on the right amount and proper functioning of other dynamin-related proteins, posttranslational modifications, and also on the mitochondria lipid cardiolipin (57). It is, therefore, likely that the ultrastructurally defective oncocytic mitochondria in an established tumor may lack the molecularly fit machinery needed for DRP1 oligomers to assemble in bigger helical-like structures, as described recently (43). Under this hypothesis, S616-p-DRP1 could be highly expressed in the early stages of tumorigenesis in an attempt to compensate for the deficient mitochondria, but once the tumor reaches the established oncocytic phenotype, the opposite phenomenon is observed. The fact that no difference between DRP1 expression in oncocytic vs. nononcocytic variants was found supports a posttranslational regulation of DRP1. The assessment of differentiation markers, such as NIS expression and iodine cell uptake, and their crosstalk with DRP1 as a key effector of mitochondrial bioenergetics and dynamic, could shed a light into the potential mechanisms of radioiodine resistance described in oncocytic thyroid tumors. The lower S616-p-DRP1 expression observed in tumors with oncocytic morphology could also explain, at least partially, why these tumors' cells are less prone to cell death. Of note, we found the same pattern a renal oncocytoma series (unpublished data). Whether this also explains radioiodine resistance itself is a question that deserves further study. Interestingly, when we treated the oncocytic TC cell line XTC.1 with a putative DRP1 inhibitor, Mdivi-1, which was shown to have anti-tumoral effects in various tumor models (10, 20, 22, 23, 42, 58), it was less sensitive to apoptosis when compared with other nononcocytic TC lines (data not shown), highlighting the innate resistance to cell death of these tumors. In the future, mechanistic studies should address the relationship between DRP1, MAPK activity, and iodine uptake in depth, aiming at redifferentiating radioiodine-resistant tumors.

We herewith hypothesize that positive expression of S616-p-DRP1 can be used as a marker of infiltrative, locally invasive tumors, and lymph node metastases. Under this assumption, the assessment of S616-p-DRP1 expression as a candidate biomarker to be used

in combination with the already established prognostic clinico-pathologic factors for pre and postoperative TC risk assessment in a larger TC patients' sample should be further explored. Due to its association with locally invasive traits and lymph node mestastases, the IHC evaluation of S616-p-DRP1 in cytology, if feasible, could be of added value when deciding the extent of surgery to be performed. Furthermore, the opposing edges of S616-p-DRP1 expression spectrum should be evaluated and its biological significance validated through mechanistic work, given the apparently paradoxical negative expression in patients with poor outcomes. To this point, the fact that the oncocytic TC variants presented the same S616-p-DRP1 expression pattern as TC with distant metastases leads us to hypothesize that S616-p-DRP1 could be one of the molecular linking pieces between two phenomenona not seldomly observed in these two clinical entities - radioiodine and programmed cell death resistance (59).

Our study is limited by its retrospective nature, which translated in limited missing data and pathological and staging classification systems used. However, this did not impact the results and our conclusions, particularly regarding the association with invasive disease. We have tested eight different definitions of DRP1- and S616-p- DRP1 positivity based on various IRS and intensity thresholds, and all have showed the same trends herewith reported (data not shown). Our results should be prospectively validated, and extended to further genotypes, including RAS- and TP53-mutated tumors. The validation of S616-p-DRP1 as prognostic factor also requires a prospectively proven significant association with hard endpoints, which is difficult to achieve given the relatively small number of patients who die due to TC.

In conclusion, both DRP1 and its activated form, S616- p-DRP1, are associated with locally invasive traits in TC. However, S616-p-DRP1 is likely a better candidate to predict locally invasive behavior of tumors, given its significant association with lymph node metastases. Therefore, validation of S616-p-DRP1 IHC assays and definition of expression scores with a clinical significance (e.g., positive vs. negative or high vs. low) should be prospectively addressed. We think that mechanistic studies should be performed exploring treatment strategies based on modeling DRP1 and MAPK activity in various TC models. This

may pave the way to further tailor treatment strategies for patients who have persistent disease, become refractory to iodine treatment, and will eventually die from distant metastization - where the unmet medical need exists.

Data availability

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Consent to participate

Samples were collected upon written informed consent of the patients, or their guardians, in case of patients under 18 years.

Ethics approval

This work was approved by the Ethic Committee for Health (CES) of the Hospital Center of São João (CHSJ)/Faculty of Medicine of the University of Porto (FMUP) (CES 137 284–13) and by the Ethic Committee of the Faculty of Medicine of the University of Coimbra (n° 1309).

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Supplementary Table 1

Odds ratio for the association between clinicopathological variables and DRP1 or S616-p-DRP1 expression

	DRP1 IRS score					S616-p-DRP1 IRS score				
Clinicopathological characteristics	**Total sample (N = 259)**	**Negative[a] (n = 90, 34.7%)**	**Positive[b] (n = 169, 65.3%)**	**OR (95% CI)**	***P* value**	**Total sample (N = 98)**	**Negative[a] (n = 81, 82.7%)**	**Positive[b] (n = 17, 17.3%)**	**OR (95% CI)**	***P* value**
Age at diagnosis, years				1.064					0.136	
≥45, n (%)	135	46 (34.1)	89 (65.9)	[0.638, 1.775]	0.812	59	53 (89.8)	6 (10.2)	[0.014, 0.754]	**0.013**
<45, n (%)	124	44 (35.5)	80 (64.5)			39	28 (71.8)	11 (28.2)		
Gender, n (%)				0.709					0.707	
Female	208	69 (33.2)	139 (66.8)	[0.378, 1.328]	0.282	73	61 (83.6)	12 (16.4)	[0.069, 3.928]	> 0.999
Male	51	21 (41.2)	30 (58.8)			25	20 (80)	5 (20)		
TNM stage, n (%)				0.773					0.311	
I+II	146	47 (32.2)	99 (67.8)	[0.462, 1.293]	0.326	44	36	8	[0.049, 1.491]	0.107
III+IV	113	43 (38.1)	70 (61.9)			54	45	9		
Tumor size				0.66					0.191	
n	251	85 (33.9)	166 (66.1)		0.224	94	79 (84)	15 (16)		0.195
≤4 cm	208	67 (32.2)	141 (67.8)	[0.337, 1.292]		74	60 (81.1)	14 (18.9)	[0, 2.135]	
>4 cm	43	18 (41.9)	25 (58.1)			20	19 (95)	1 (5)		
Invasion, n (%)										
Vascular										
n	223	77 (34.5)	146 (65.5)	0.968	0.912	79	65 (82.3)	14 (17.7)	0.590	0.490
Present	80	28 (35)	52 (65.0)	[0.545, 1.72]		37	30 (81.1)	7 (18.9)	[0.08, 2.739]	
Absent	143	49 (34.3)	94 (65.7)			42	35 (83.3)	7 (16.7)		
Extrathyroidal				1.707					2.935	
n	227	79 (34.8)	148 (65.2)		0.072	80	65 (81.2)	15 (18.8)		0.180

	Present	84	23 (27.4)	61 (72.6)	[0.951,		38	27 (71.1)	11 (28.9)	[0.6, 18.785]	
	Absent	143	56 (39.2)	87 (60.8)	3.065]		42	38 (90.5)	4 (9.5)		
Multifocality, n (%)					1.377					1.188	
n		228	79 (34.6)	149 (65.4)		0.326	80	65 (81.2)	15 (18.8)		> 0.999
	Present	83	25 (30.1)	58 (69.9)	[0.773,		37	28 (75.7)	9 (24.3)	[0.248,	
	Absent	145	54 (37.2)	91 (62.8)	2.453]		43	37 (86)	6 (14)	5.654]	
Lymph node metastases					0.852					15.594	
n		257	88 (34.2)	169 (65.8)		0.555	97	80 (82.5)	17 (17.5)		**0.012**
	Present	93 (36.2)	34 (36.6)	59 (63.4)	[0.5, 1.452]		60	46 (76.7)	14 (23.3)	[1.569, Inf]	
	Absent	164 (63.8)	54 (32.9)	110 (67.1)			37	34 (91.9)	3 (8.1)		
Distant metastases					0.629					1.304	
n		257	90 (34.9)	168 (65.1)		0.212	97	97 (89.7)	10 (10.3)		0.668
	Present	35 (13.6)	16 (45.7)	19 (54.3)	[0.302,		16	14 (87.5)	2 (12.5)	[0.122,	
	Absent	223 (86.4)	74 (33.2)	149 (66.8)	1.308]		81	73 (90.1)	8 (9.9)	7.556]	
Molecular diagnosis, n (%)											
***BRAF*^{V600E} mutation**											
n		196	68 (34.9)	128 (65.1)	1.383	0.287	67	57 (85.1)	10 (14.9)	0.521	0.495
	Positive	88	27 (39.7)	61 (47.7)	[0.761, 2.512]		36	32 (88.9)	4 (11.1)	[0.098, 2.497]	
	Negative	108	41 (60.3)	67 (39.7)			31	25 (80.6)	6 (19.4)		
***TERT* promotor mutation**					0.581					0.238	
n		187	68 (36.4)	119 (63.6)		0.222	66	50 (75.8)	16 (24.2)		0.334
	Positive	23	11 (47.8)	12 (52.2)	[0.241,		9	8 (88.9)	1 (11.1)	[0, 2.593]	
	Negative	164	57 (34.8)	107 (65.2)	1.399]		57	42 (73.7)	15 (26.3)		
Disease-free status at the end of follow-up, n (%)						0.091					
n		236	78 (33.1)	158 (66.9)	1.652		90	73 (81.1)	17 (18.9)	0.723	> 0.999
	Yes	168	50 (29.8)	118 (70.2)	[0.92, 2.966]		57	47 (82.5)	10 (17.5)	[0, 8.261]	
	No	68	28 (41.2)	40 (58.8)			33	26 (78.8)	7 (21.2)		

Abbreviations: CI, confidence interval; DRP1, dynamin-related protein 1; IRS, immune-reactive staining; Neg, negative; OR, odds ratio; p-, Phospho-; Pos, positive; S, serine. S616-p-DRP1, Serine 616-phosphorylated DRP1. Bold values indicate the result was statistically significant.

(a) negative DRP1 expression was defined as IRS <6

(b) positive DRP1 expression was defined as IRS ≥6

Table 2

Characteristics of patients and tumors

Clinicopathological characteristics	DRP1 IRS score			S616-p-DRP1 IRS score		
	Total sample (N = 259, 100%)	**Negative[a] (n = 90, 34.7%)**	**Positive[b] (n = 169, 65.3%)**	**Total sample (N = 98)**	**Negative[a] (n = 81, 82.7%)**	**Positive[b] (n = 17, 17.3%)**
Age at diagnosis, years						
≥45, n (%)	135 (52.1)	46 (34.1)	89 (65.9)	59	53 (89.8)	6 (10.2)
<45, n (%)	124 (47.9)	44 (35.5)	80 (64.5)	39	28 (71.8)	11 (28.2)
Mean ± SD	45.5 ± 16.3	45.9 ± 17.8	45.3 ± 15.5	48.1 ± 16.5	49.8 ± 16.1	39.9 ± 17.4
Gender, n (%)						
Female	208 (80.3)	69 (33.2)	139 (66.8)	73	61 (83.6)	12 (16.4)
Male	51 (19.7)	21 (41.2)	30 (58.8)	25	20 (80)	5 (20)
Histological diagnosis, n (%)						
PTC	162 (62.5)	47 (29)	115 (71.0)	66	58 (87.9)	8 (12.1)
FVPTC	63 (24.3)	29 (46)	34 (54.0)	13	11 (84.6)	2 (15.4)
FTC	25 (9.7)	9 (36)	16 (64.0)	13	13 (100)	0 (0)
PDTC	9 (3.5)	5 (55.6)	4 (44.4)	6	5 (83.3)	1 (16.7)
Oncocytic variants, n (%)[a]						
n	247	85 (34.4)	162 (65.6)	94	77 (81.9)	17 (18.1)
Yes	32 (13.0)	9 (28.1)	23 (71.9)	29	29 (100)	0 (0)
No	215 (87.0)	76 (35.3)	139 (64.7)	65	48 (73.8)	17 (26.2)
TNM stage, n (%)						
I	122 (47.1)	39 (32)	83 (68.0)	39	31 (79.5)	8 (20.5)
II	24 (9.3)	8 (33.3)	16 (66.7)	5	5 (100)	0 (0)
III	54 (20.8)	18 (33.3)	36 (66.7)	23	19 (82.6)	4 (17.4)
IV	59 (22.8)	25 (42.4)	34 (57.6)	31	26 (83.9)	5 (16.1)
Tumor size						
n	251	85 (33.9)	166 (66.1)	94	79 (84)	15 (16)
Mean ± SD, cm	27.2 ± 16.4	28.9 ± 18.1	25.8 ± 15.3	29.9 ± 17.8	31.3 ± 18.3	23.0 ± 7.3

≤4 cm	208 (82.9)	67 (32.2)	141 (67.8)	74	60 (81.1)	14 (18.9)
>4 cm	43 (17.1)	18 (41.9)	25 (58.1)	20	19 (95)	1 (5)
Encapsulated tumors, n (%)						
n	215	74 (34.4)	141 (65.6)	79	65 (82.3)	14 (17.7)
Yes	108(50.2)	46 (42.6)	62 (57.4)	41	38 (92.7)	3 (7.3)
No	107(49.8)	28 (26.2)	79 (73.8)	38	27 (71.1)	11 (28.9)
Invasion, n (%)						
Tumor capsule						
n	100	45 (45)	55 (55.0)	35	32 (91.4)	3 (8.6)
Yes	80 (80.0)	36 (45)	44 (55.0)	30	27 (90)	3 (10)
No	20 (20.0)	9 (45)	11 (55.0)	5	5 (100)	0 (0)
Vascular						
n	223	77 (34.5)	146 (65.5)	79	65 (82.3)	14 (17.7)
Present	80 (35.9)	28 (35)	52 (65.0)	37	30 (81.1)	7 (18.9)
Absent	143(64.1)	49 (34.3)	94 (65.7)	42	35 (83.3)	7 (16.7)
Thyroid capsule						
n	222	77 (34.7)	145 (65.3)	79	64 (81)	15 (19)
Present	109 (49.1)	29 (26.6)	80 (73.4)	47	33 (70.2)	14 (29.8)
Absent	113(50.9)	48 (42.5)	65 (57.5)	32	31 (96.9)	1 (3.1)
Extrathyroidal						
n	227	79 (34.8)	148 (65.2)	80	65 (81.2)	15 (18.8)
Present	84 (37.0)	23 (27.4)	61 (72.6)	38	27 (71.1)	11 (28.9)
Absent	143 (63.0)	56 (39.2)	87 (60.8)	42	38 (90.5)	4 (9.5)
Multifocality, n (%)						
n	228	79 (34.6)	149 (65.4)	80	65 (81.2)	15 (18.8)
Present	83 (36.4)	25 (30.1)	58 (69.9)	37	28 (75.7)	9 (24.3)
Absent	145(63.6)	54 (37.2)	91 (62.8)	43	37 (86)	6 (14)
Lymph node metastases, n (%)						
n	257	88 (34.2)	169 (65.8)	97	80 (82.5)	17 (17.5)
Present	93 (36.2)	34 (36.6)	59 (63.4)	60	46 (76.7)	14 (23.3)
Absent	164 (63.8)	54 (32.9)	110 (67.1)	37	34 (91.9)	3 (8.1)
Distant metastases, n (%)	258	90 (34.9)	168 (65.1)	97	97 (89.7)	10 (10.3)

n						
Present	35 (13.6)	16 (45.7)	19 (54.3)	16	14 (87.5)	2 (12.5)
Absent	223 (86.4)	74 (33.2)	149 (66.8)	81	73 (90.1)	8 (9.9)
Molecular diagnosis, n (%)						
***BRAF*^{V600E} mutation**						
n	215	75 (34.9)	140 (65.1)	76	59 (77.6)	17 (22.4)
Positive	88 (40.9)	27 (30.7)	61 (69.3)	36	30 (83.3)	6 (16.7)
Negative	127 (59.1)	48 (37.8)	79 (62.2)	40	29 (72.5)	11 (27.5)
***TERT* promotor mutation**						
n	187	68 (36.4)	119 (63.6)	66	50 (75.8)	16 (24.2)
Positive	23 (12.3)	11 (47.8)	12 (52.2)	9	8 (88.9)	1 (11.1)
Negative	164 (87.7)	57 (34.8)	107 (65.2)	57	42 (73.7)	15 (26.3)
Radioiodine treatment, n (%)						
Yes	219 (84.6)	73 (33.3)	146 (66.7)	83	66 (79.5)	17 (20.5)
No	40 (15.4)	17 (42.5)	23 (57.5)	15	15 (100)	0 (0)
No. of doses, n (%)						
n	219	73 (33.3)	146 (66.7)	82	65 (79.3)	17 (20.7)
1	140 (63.9)	39 (27.9)	101 (72.1)	44	36 (81.8)	8 (18.2)
≥2	79 (36.1)	34 (43)	45 (57.0)	38	29 (76.3)	9 (23.7)
Cumulative iodine dosage,						
n	241	84 (34.9)	157 (65.1)	91	74 (81.3)	17 (18.7)
mean ± SD, mCi	195.1 ± 235.1	230.0 ± 285.5	176.5 ± 201.6	223.9 ± 230.1	202.5 ± 217.7	317.4 ± 265.0
Disease-free status at the end of follow-up, n (%)						
n	236	78 (33.1)	158 (66.9)	90	73 (81.1)	17 (18.9)
Yes	168 (71.2)	50 (29.8)	118 (70.2)	57	47 (82.5)	10 (17.5)
No	68 (28.8)	28 (41.2)	40 (58.8)	33	26 (78.8)	7 (21.2)
Follow-up time, mean ± SD, years	8.6 ± 6.4	9.4 ± 6.5	8.2 ± 6.4	8.1 ± 6.0	6.6 ± 6.0	8.8 ± 5.1
Overall mortality	17 (6.6)	9 (52.9)	8 (47.1)	9	8 (88.9)	1 (11.1)
Disease-related mortality	9 (3.5)	3 (33.3)	6 (66.7)	5	5 (100)	0 (0)

BRAF, B-Raf proto-oncogene; DRP1, dynamin-related protein 1; FTC, follicular thyroid carcinoma; FVPTC, follicular variant of papillary thyroid carcinoma; IRS, immune-reactive staining; mCi, millicurie; n, number of cases; PDTC, poorly differentiated thyroid carcinoma; Pos, positive; PTC, papillary thyroid carcinoma; SD, standard deviation; S616-p-DRP1, Serine 616-phosphorylated DRP1; TERT, telomerase reverse transcriptase; TNM, tumor, node and metastasis

(a) negative DRP1 expression was defined as IRS <6

(b) positive DRP1 expression was defined as IRS ≥6

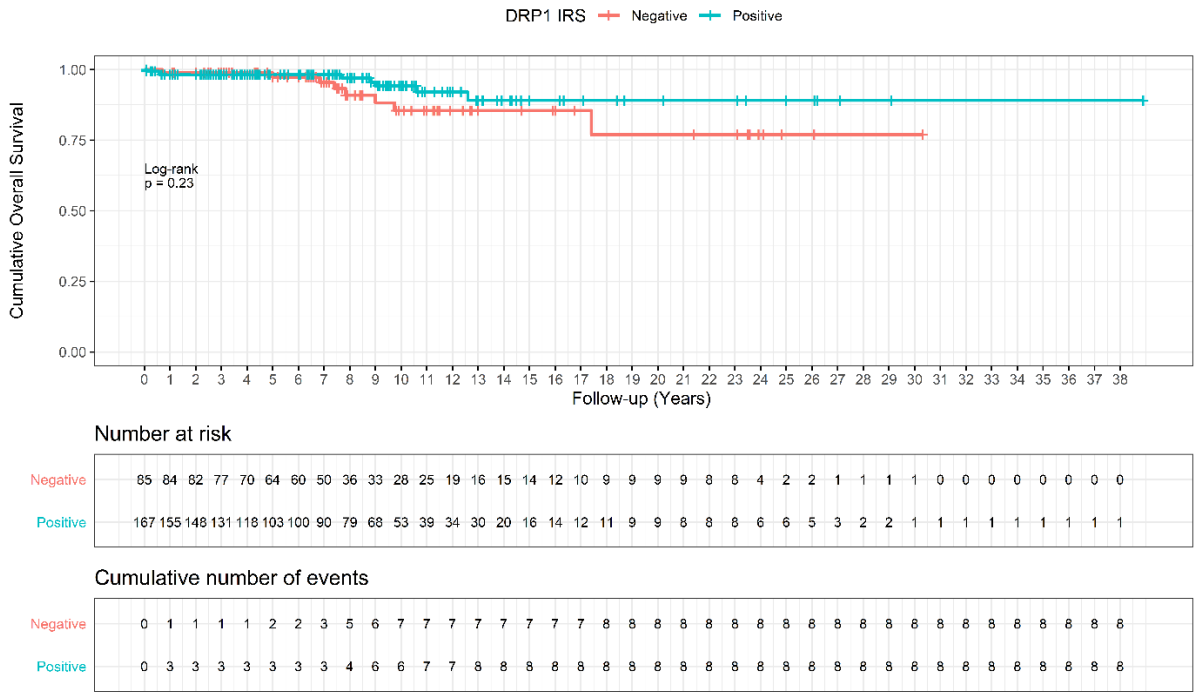
Supplementary Table 2

DRP1 and S616-p-DRP1 expression and survival outcome of patients who presented distant metastases[a]

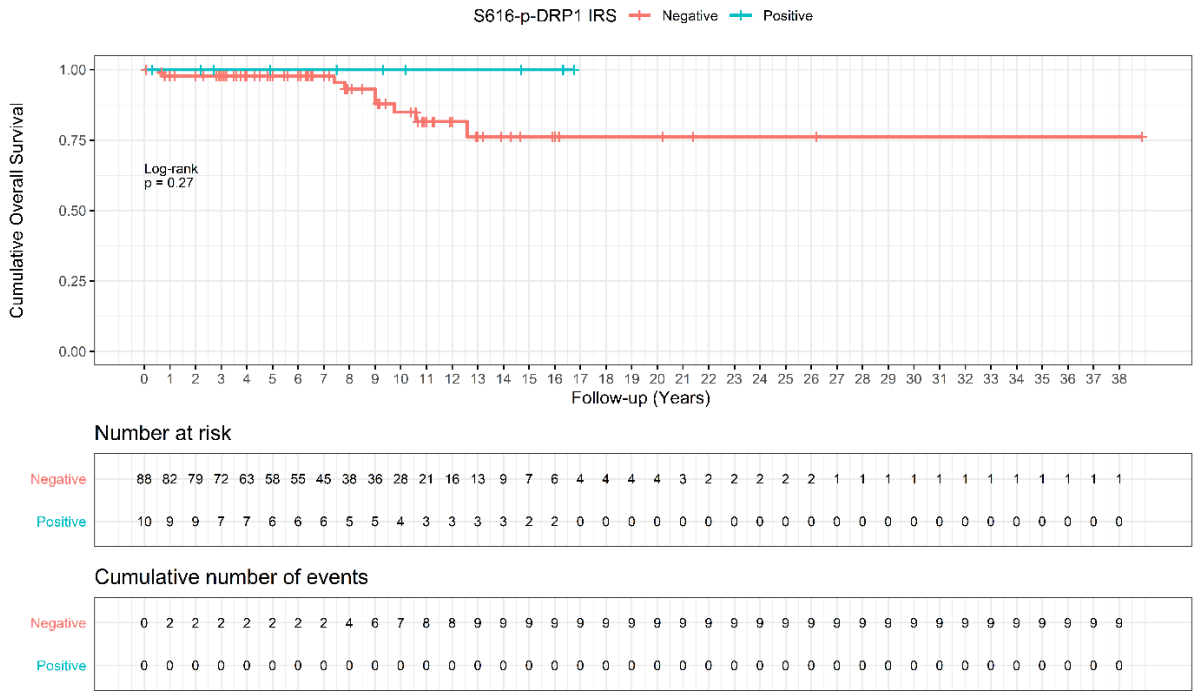
Patient number	TC type	IRS score		Survival outcome	Follow-up duration (years)
		DRP1	S616-p-DRP1		
1	tcPTC	8	3	Died	9.0
2	cPTC	12	0	Died	10.6
3	PDTC	3	0	Died	7.4
4	sPTC	2	0	Died	0.7
5	PDTC	8	0	Died	12.6
6	oPDTC	4	0	Alive	6.4
7	PDTC	3	0	Alive	21.4
8	tcPTC	9	2	Alive	1.0
9	cPTC	4	0	Alive	7.8
10	cPTC	4	0	Alive	2.0
11	cPTC	9	0	Alive	26.2
12	cPTC	3	4	Alive	9.0
13	cPTC	9	2	Alive	2.3
14	cPTC	6	6	Alive	10.2
15	cPTC	8	6	Alive	2.0
16	FTC	8	0	Alive	2.9

Abbreviations: cPTC, classic papillary carcinoma; DRP1, dynamin related protein 1; IRS, immune-reactive staining; mPTC, mixed papillary carcinoma; oPDTC, oncocytic variant of poorly differentiated carcinoma; PDTC, poorly differentiated thyroid carcinoma; S616-p-DRP1, Serine 616-phosphorylated DRP1; stPTC, solid/trabecular variant of papillary carcinoma; tcPTC, tall cell variant of papillary carcinoma; TC, thyroid carcinoma.

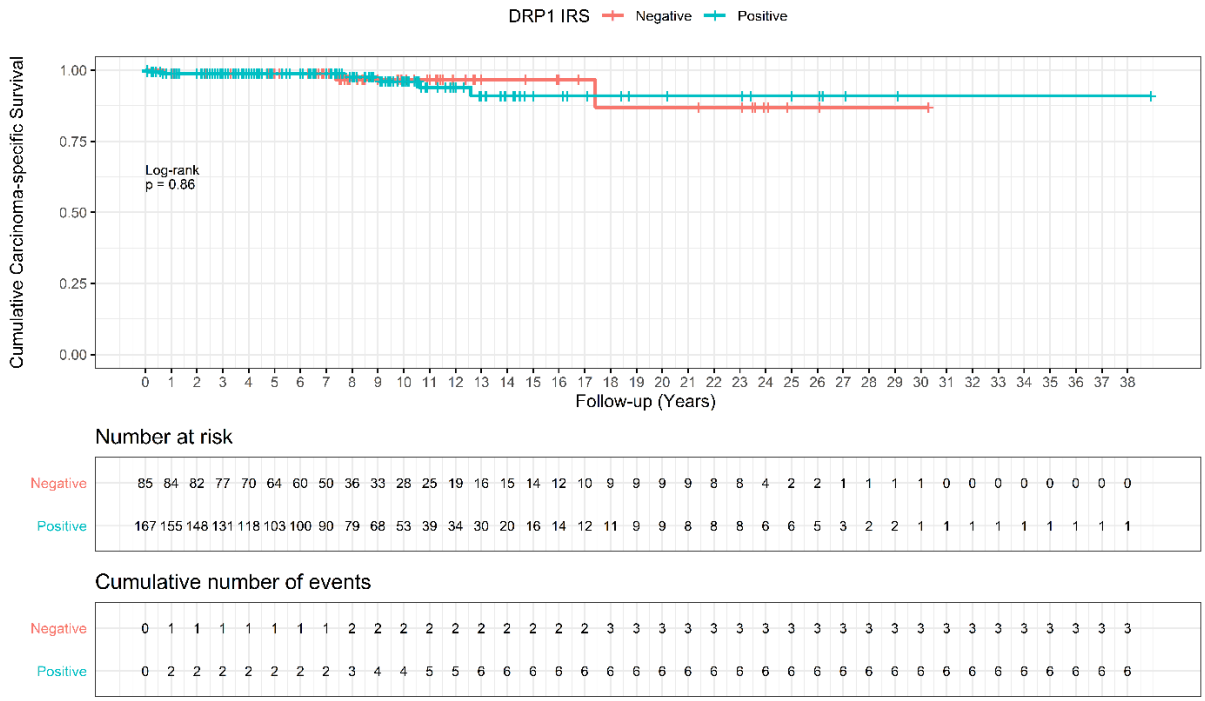
(a) the analysis was restricted to tissue samples tested both for DRP1 and S616-p-DRP1 immunostaining



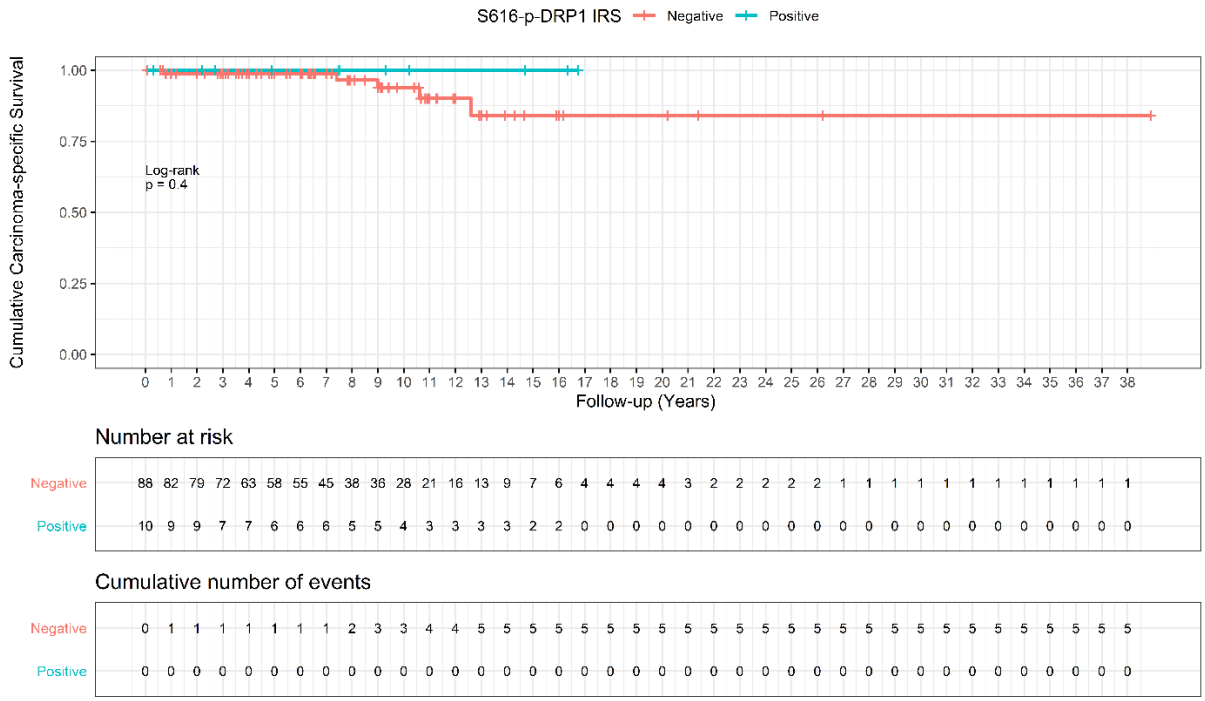
Supplementary Fig. 1 Kaplan-Meier overall survival curves of patients stratified by negative or positive expression of DRP1. Red lines represent the negative DRP1 expression (defined as IRS <6) group and the blue lines represent the positive DRP1 expression (defined as IRS ≥6) group. Log-rank test was used to statistically compare the curves and *P* value is shown. DRP1, dynamin related protein 1; IRS, immune-reactive staining



Supplementary Fig. 2 Kaplan-Meier overall survival curves of patients stratified by negative or positive expression of S616-DRP 1. Red lines represent the negative S616-DRP1 expression (defined as IRS <6) group and the blue lines represent the positive S616-DRP1 expression (defined as IRS ≥6) group. Log-rank test was used to statistically compare the curves and *P* value is shown. DRP1, dynamin-related protein 1; IRS, immune-reactive staining; S616-DRP1, Serine 616-phosphorylated DRP1



Supplementary Fig. 3 Kaplan-Meier carcinoma-specific survival curves of patients stratified by negative or positive expression of DRP1. Red lines represent the negative DRP1 expression (defined as IRS <6) group and the blue lines represent the positive DRP1 expression (defined as IRS ≥6) group. Log-rank test was used to statistically compare the curves and P value is shown. DRP1, dynamin-related protein-1; IRS, IRS, immune-reactive staining



Supplementary Fig. 4 Kaplan-Meier carcinoma-specific survival of patients stratified by negative or positive expression of S616-p-DRP1. Red lines represent the negative p-DRP1 expression (defined as IRS <6) group and the blue lines represent the positive S616-p-DRP1expression (defined as IRS ≥6) group. Log-rank test was used to statistically compare the curves and P value is shown. DRP1, dynamin-related protein 1; IRS, IRS, immune-reactive staining; S616-p-DRP1, Serine 616-phosphorylated DRP1

5. Prognosis potential of dynamin-related protein 1 as a biomarker for differentiated thyroid carcinoma risk assessment

The hypothesis that DRP1 could have a prognosis potential in the management of DTC stems from different reports already summarized by our group, and in this dissertation Introduction, which link its expression and/or activation to various hallmarks of cancer (119). However, there are two particular published reports which represent the basis for our thinking:

Weider *et al* described the expression of S616-p-DRP1 in 48.7% melanoma patients-derived samples, 95.6% of which were *BRAF*^{V600E} mutation positive tumors, with only 6.8% expression in *BRAF*^{Wt} (120). Interestingly, the same relationship with *BRAF* mutational status was observed in 40 cases of dysplastic nevi, where 79.3 % of stained positively for phosphorylated S616-p-DRP1 were also *BRAF*^{V600E} (120). Similarly, 92% of *BRAF*^{V600E} samples were positive for S616-p-DRP1. Matched benign and dysplastic nevi cohort of 46 samples showed the same significant association, however the strongest correlations were found in *BRAF*^{V600E} melanoma (120). The authors used A375 *BRAF*^{V600E} melanoma cell line to genetically and pharmacologically inhibit DRP1 using iRNA lentivirus and Mdivi-1, respectively (120). The genetic inhibition of DRP1 led to the loss of DRP1 expression and a decrease in cell proliferation. The pharmacological inhibition led to a decrease in DRP1 dependent mitochondrial fission and to a dose-dependent apoptosis, which was not seen in the control wild-type-*BRAF* (*BRAF*^{Wt}) cell line, suggesting that the induction of phosphorylated S616-p-DRP both in dysplastic nevi and in primary melanoma has a role in the *BRAF*^{V600E}-driven disease, which raised the hypothesis about the potential role of S616-p-DRP as a prognosis biomarker in addition to BRAF to identify which lesions will most likely develop melanoma (120). An important point is that of S616-p-DRP was not expressed in normal skin, just like it was not express in our series, in normal thyroid tissue.

Tanwar *et al* have published on an exploratory analysis of gene expression data from the 31 cancer types in TCGA, showing that DRP1 is predominantly co-expressed with genes involved in cell cycle and those involved in gene expression and metabolism, across majority of the cancer types (121). The authors have specifically studied epithelial ovarian cancer (EOC) due to previous work showing that the mitotic transcription factor forkhead box protein M1 (FOXM1) driven cell cycle pathway is altered in these patients, and based on their own previous observation of an active role of DRP1 in ovarian epithelial cell layer development in Drosophila (122, 123). Based on the TGCA findings and on the observation that three different EOC patient cohorts – chemotherapy sensitive, recurred-resistant and resistant - had different DRP1 expression profiles. The recurred-resistant patients had a higher DRP1 expression in their primary tumors, and the primary resistant tumors had an even higher DRP1 expression in their primary tumors, compared with the sensitive tumors. What is even more interesting is that, unlike what was observed in the chemotherapy-resistant tumors, in the sensitive and the recurred-resistant tumors DRP1 co-expressed with cell cycle genes. The authors suggest that DRP1 may have a pro-apoptotic role in DRP1-Low and an anti-apoptotic role in DRP1-High patients, and they finally proposed that a DRP1-based-gene expression-signature from their primary tumors could potentially identify EOC patients who may have better or worse survival after exposure to the standard platinum-taxane based chemotherapy (121).

It is recognized that thyroid tissue is a conditionally renewing tissue, which proliferates rarely in adult life (6). Nevertheless, upon a defined genomic and (micro)environmental context apoptosis is evaded, uncontrolled proliferation is triggered along with more or less phenotypic evidence of the escape to normal cell self-regulation mechanisms. To this point, DRP1 expression pattern is the evidence of these self-regulation mechanisms, particularly in the context of tumorigenesis, given the plead of cellular processes where it is involved in the normal cell and in the transformed cell (119).

Our IHC data show that total DRP1 is not as discretionary in TC as we would have anticipated, based on the described tumor models. DRP1 is positive in a relatively high proportion of tumors - 65.3% -, it associates with general indicators of tumor local invasion, but it does not clearly dichotomize patients who will have a better prognosis from those who will have an unfavorable outcome. S616-p-DRP1, on the other hand, was positive in 17.3% of the tumors, and was more significantly associated with locally invasive disease, including lymph node metastization. More importantly, the majority of patients who presented distant metastases (14/16) were negative for the expression of S616-p-DRP1, of whom 5 patients died due to TC. Intriguingly, unlike what was reported for melanoma, we did not find any association with *BRAF*^{V600E} mutation. We could explain this result by the smaller sample size tested for S616-p-DRP1, where only 36 patients were positive for *BRAF*^{V600E}, but based on the strong correlation which was found in dysplastic nevi, and particularly in melanoma, it is unlikely that we will see any different results in a bigger TC sample. On the contrary, the sample size factor may explain and fit better with the lack of association seen with *TERT* promoter mutations, which was present in only 9 of the tumor samples tested for S616-p-DRP1. Interestingly, all *TERT* promoter mutated-tumors were negative for S616-p-DRP1. This trend in our results is consistent with the already described association between *TERT* promoter mutations and distant metastases (50).

Based on these data, we propose that the assessment of the expression of S616-p-DRP1 may proof to be a strong negative predictor of the risk of distant metastases and death, although still weighting in other established clinico-pathological factors that support DTC prognosis. Under these same assumptions, we also suggest that S616-p-DRP1 may also support the assessment of DTC risk of recurrence, in which case it may be a positive predictive biomarker of locally invasive behavior. A detailed description of how this could be clinically implemented is further described below.

In the assessment of DTC risk, one needs to consider both the risk of death due to the disease and the risk of disease recurrence. The risk of death is most accurately assessed by the AJCC/TNM system (124), while the risk of disease recurrence is based on the ATA stratification system (23). The former takes into consideration the size of the tumor as well as lymph node and distant metastization and is still considered to be the best predictor of disease-related mortality. The risk of recurrence stratification, on the other hand, includes a vaster number of clinico-pathological characteristics, such as tumor histology – including its different variants -, vascular invasion, micro or macroinvasion invasion of perithyroidal soft tissue, evidence of lymph node or distant metastases, tumor-related symptoms, molecular markers – *BRAF* and/or *TERT* promoter mutations -, surgical tumor resection completeness and post-therapy thyroglobulin values. The risk of disease recurrence is critical for the therapeutic approach, not only in terms of the surgical extent, but also to support the need for RAI, its doses, and TSH suppression. As already mentioned, this risk assessment should be revised during the follow-up of the patient, when new clinical information is available, tailoring further therapeutic interventions and patient follow-up strategy (3, 23).

Based on our data, we have found that S616-p-DRP1 positive expression is significantly higher in tumors with locally invasive pattern and with lymph node metastases. Unarguably, the identification of molecular biomarkers which can be early predictors of poor outcomes is more relevant in the context of DTC, where the 10-year survival rates surpass 90% to 95%, and only a relatively small number of patients will evolve with persistent disease and/or who will eventually present distant metastases, develop resistance do RAI and possibly die due to TC (59). If we would be able to identify those patients as early as possible in the treatment algorithm, this would allow us to better tailor treatment and follow-up strategies for this particular patient sub-group. Ideally, we should be able to test the expression of S616-p-DRP1 in the pre-operative context, in a cytology sample, to better stratify the risk of recurrence and therefore the surgical extent under the context of clinical aspects such as the age, clinical signs and

symptoms, and imaging assessment. The need for more personalized assessment in the post-operative setting of the risk becomes obvious by the evidence that the risk of death from TC is not accompanied by the risk of recurrence, particularly in young patients (< 55 years old) with stage I disease, as per the AJCC/IUAC 8th edition staging system (Table 3) (10). This is a diverse group which includes patients presenting a very low risk of recurrence and patients with a high risk of recurrence (3, 23). The assessment of S616-p-DRP1 expression through IHC may help further characterize the invasive nature of the primary tumor and support the decision of adopting a more intensive surgical approach, with completion of thyroidectomy following lobectomy, and possibly with prophylactic central node dissection, based on the above mentioned clinico-pathological aspects. This may be particularly useful for those low risk cases (T1b, T2 N0), where we could more accurately identify the patients that will benefit from these strategies with the aim of reducing recurrence rate. If we consider the ATA risk stratification system and the absence of evident signs which can predict disease recurrence in the group of low-risk patients (5% or less risk of disease recurrence), the integration of a molecular biomarker such as S616-p-DRP1 could be of potential interest, given the association of its expression with a locally invasive behavior and lymph node metastases. In this particular group of patients - with no macroscopic tumor-tissue remnants post-surgery or signs of locoregional and distant metastases in a classic PTC, *BRAF*^{V600E}-negative, with no vascular invasion, or with an intrathyroidal FTC with no vascular invasion or just minimal angioinvasion -, S616-p-DRP1 could help identify those patients who may be eligible for a more radical surgical approach and particularly for adjuvant RAI. Going back to the most recent 8th AJCC/IUAC TNM staging edition (Table 3), older patients (> 55 years old) with metastatic involvement of central or lateral neck lymph nodes or with gross extrathyroidal involvement of overlying strap muscles are staged as II disease. These are patients with a lower disease-specific survival as compared to stage I. In stage II, particularly for T3a (>4 cm), N0/Nx tumors, one might consider that a biomarker which can predict a higher invasive behavior, such as lymph node involvement, could be useful.

If we apply the same thinking to the intermediate risk group of the ATA risk stratification system, we could also propose the use of S616-p-DRP1 to be included in the assessment of those cases which are in the lower limit of the recurrence risk within this group (3%–8% of risk of disease recurrence), classified as such based on the evidence of microscopic invasion of perithyroidal soft tissues (3, 23).

However, as we advance in the staging and in the risk stratification systems, the usefulness of a biomarker such as S616-p-DRP1 is less obvious, since other important histopathologic and molecular prognostic factors become more evident. We now have more high-risk patients in stage III when compared to same stage in the previous 7th edition. Stage III is composed of patients with gross extrathyroidal extension into major structures in the neck, with no distant metastases at diagnosis, but also includes all patients with lateral neck lymph node involvement which are not considered as having a high risk of death due to TC (3, 23). For this reason, the latter were excluded from stage IV in the current AJCC/UIAC TNM staging edition (3, 23). In stage III disease and/or in cases of intermediate risk disease per ATA classification, S616-p-DRP1 testing might prove to be of value as a negative predictor of poor outcomes. That is, if tumors would be classified as negative for S616-p-DRP1 expression, the likelihood of a poor outcome could be higher. Indeed, based on the trend observed in our IHC series, where 14 out of the 16 patients with distant metastases stained negative of S61-p-DRP1, including all 5 patients who died due to TC. However, the hypothesis that a negative S616-p-DRP1 expression could be a predictor of poor prognosis, i.e. a biomarker with a negative predictive value, is one that needs validation.

Based on our data, we cannot tell whether the negative S616-p-DRP1 expression pattern is just the phenotypic expression of less differentiated tumors - and therefore the link to poor outcomes -, as our DRP1 IHC data seem to indicate, or if there is a mechanistic explanation behind this finding. We know that, based on other tumor models such as laryngeal carcinoma, the mitochondrial deoxyribonucleic acid (mtDNA) content as a marker of higher genomic oxidative stress predicts poor outcomes in early stage

disease (125). If we assume that the activation of DRP1 is a response to a defective OXPHOS process and/or to oxidative stress, the hypothesis could be that S616-p-DRP1 would be linked with a poor prognosis, which does not support the idea that a negative expression associates with poor outcomes.

Table 3

A clinically based approach to staging in differentiated thyroid cancer using the 8th edition AJCC/IUAC TNM update (*adapted from (126)*).

	Distant metastases	Gross ETE present	Structures involved with gross ETE	T category	N category	Stage
<55 years	No	Yes or No	Any or None	Any	Any	I
	Yes	Yes or No	Any or None	Any	Any	II
≥55 years	No	No	None	≤4 cm	N0/Nx	I
				(T1-T2)	N1a/N1b	II
				>4 cm (T3a)	N0/Nx/N1a/N1b	II
		Yes	Only strap muscle (T3b)	Any	Any	II
			Subcutaneous, larynx, trachea, esophagus, recurrent laryngeal nerve (T4a)	Any	Any	III
			Prevertebral fascia, encasing major vessels (T4b)	Any	Any	IV4A
	Yes	Yes or No	Any or None	Any	Any	IV4B

6. Dynamin-related protein 1 as a therapeutic target in follicular cell-derived thyroid carcinoma

Various studies have reported on the antitumoral effect of the pharmacological and genetic inhibition of DRP1. The fission pattern, associated with an increased expression of DRP1, or with its activation, is one observed in most of the tumor models explored so far, which was covered in our review paper (119). The same is true for some of the most prevalent neurodegenerative or metabolic diseases, such as Alzheimer's disease and type 2 diabetes, respectively (127, 128). In theory, the inhibition of DRP1 as the main orchestrator of mitochondrial fission would be more feasible than the promotion of mitochondrial fusion, where multiple molecular targets are involved, although this may be an oversimplified view. From this perspective, DRP1 seems to be an attractive pharmacological target, and even more so if its expression proves to be correlated with clinical outcomes in a particular disease model.

We have used two compounds directed to two different molecular targets in our mechanistic cell line. The first, Mdivi-1, has been used *in vitro* and *in vivo* in various tumor models and in neurodegenerative diseases and ischemia-reperfusion injury models as a putative DRP1 inhibitor (129-131). Mdivi-1 is a quinazolinone derivative identified in a chemical library screen, in 2008, as a GTPase inhibitor of the yeast homolog of DRP1, Dnm1 (132). Although its mechanism of action is not yet fully clarified, namely the potential off-target effects pointed by some authors (133, 134), its consistent effects on mitochondrial size, cell proliferation, apoptosis, invasion, migration and metabolism, mostly correlated with DRP1 genetic inhibition, supported our decision to use it as DRP1 inhibitor (112, 135-146). Mdivi-1 also had additional advantages which could be clinically relevant. It has shown *in vivo* activity in a lung cancer model (139), it showed no toxicity in mouse models of in doses (130, 131, 147, 148), and it is able to cross blood-brain barrier (130, 131). Additionally, DRP1 inhibition did not show effect in mouse embryonic

fibroblasts (MEF) and mouse embryonic stem (ES) cells (149). The second compound is dabrafenib, a potent and reversible ATP-competitive inhibitor of BRAF kinase, selective for *BRAF*^{V600E}-, as well as *BRAF*^{V600K}-mutated monomers, in *vitro* and *in vivo* (108, 150). The basis of our choice for dabrafenib was two-ponged: first, constitutive activation of BRAF by V600E mutation is the most frequent mutation in DTC, particularly in PTC, as described earlier (27). It has been associated with a worse prognosis in some series of patients with PTC and with loss of RAI avidity in recurrent PTC (28-35). Second, dabrafenib not only showed tumor inhibition in a xenograft model of *BRAF*-mutant PTC - with downregulation of downstream targets of MAPK pathway -, but more importantly, it stimulated RAI uptake in 6/10 (60%) patients with metastatic *BRAF*^{V600E}-mutant iodine-refractory PTC, as reported by Rothenberg *et al* (39).

The concept of associating a mitochondrial-targeted therapy with a MAPK inhibitor is based on a serious of reports suggesting that the former could potentially serve as a treatment strategy to overcome resistance to both chemotherapy and MAPK inhibitors (138, 151-153). Additionally, as already described, there is clear evidence that a close cross-talk between DRP1 and the MAPK pathway is established under normal and tumoral conditions (136, 154-156). Indeed, mitochondrial division seems to be a requisite to RAS-induced transformation and it is also a target of MAPK inhibition (136).

As described in our Introduction, it is clear that the currently available TKIs, namely sorafenib and lenvatinib, have short-lived effects, with no impact on OS. Therefore, new strategies are sought to provide therapeutic added value in the iodine-refractory patient population which patients will eventually become resistant to the effects of a MAPK inhibitor. Reverting patients' iodine-sensitiveness would possibly be the best strategy to achieve disease control or remission, and the clinical data reported with dabrafenib and selumetinib were encouraging in this respect (39, 40). This clinical unmet need, as well as the evidence already described, supported the rationale for combining both Mdivi-1 and dabrafenib in the cell line assays we have done.

Based on the IHC data we have discussed, it is our belief that targeting mitochondrial fission through DRP1 may prove to be beneficial in some clinical circumstances. Given the complexity of the DRP1 role under normal cell conditions, and even more so under a malignant cell context, we will summarize the evidence that can support different treatment strategies based on the various DTC hystotypes, including the oncocytic variants, and the recently renamed HCC (16). In the end of this chapter a summary table is provided (Table 4).

According to the gene expression data for 496 patients with PTC in TGCA database, these tumors can be dichotomized in BRAF-like (BUL), having a lower thyroid differentiation score (TDS), and RAS-like (RL), with a higher TDS. Patients with PTC who are *BRAF*-mutated, and eventually become refractory to iodine treatment, could potentially benefit from a combination of a BRAF inhibitor, such as dabrafenib, and Mdivi-1. This thesis is based on the evidence from our cell line work in which Mdivi-1 is generally more effective in reducing cell viability than dabrafenib alone, including in 8505C cell line, which harbors the $BRAF^{V600E}$ mutation (Figure 1, C).

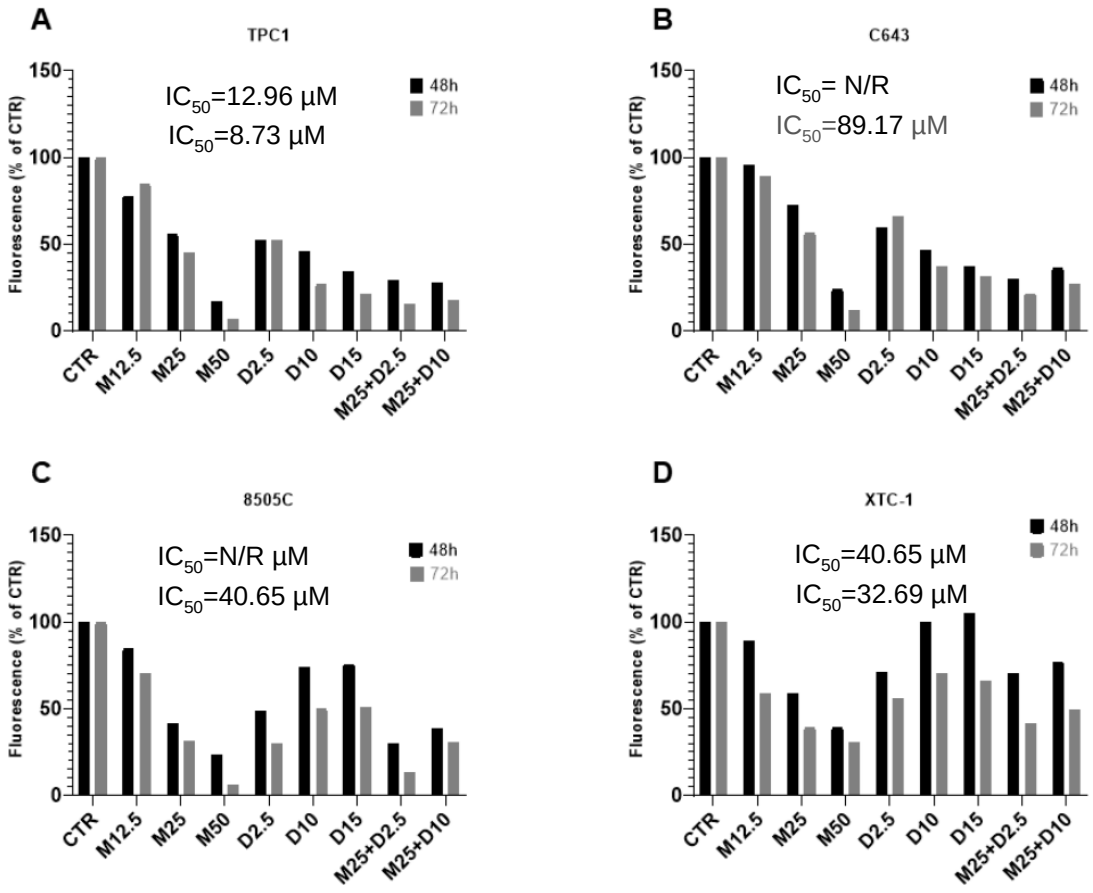


Figure 1. Comparison of the effects of treatment with Mdivi-1, Dabrafenib and Mdivi-1 and Dabrafenib combinations in thyroid cell lines viability.

Graphic representation, through a bar chart, of the percentage of TPC1 (A), C643 (B), 8505C (C) and XTC-1 (D) cells emitting fluorescence after treatment with Mdivi-1 (represented as M) – 12.5 µM, 25 µM and 50 µM -, with Dabrafenib (represented as D) – 2.5 µM, 10 µM and 15 µM – and with Mdivi-1 and dabrafenib combinations – Mdivi-1 25 µM plus dabrafenib 2.5 µM and Mdivi-1 25 µM plus dabrafenib 10 µM -, during 48h and 72h, determined by cell viability Sulforhodamine B assay, regarding to the cell fluorescence in the control (CTR) – DMSO added to cell culture medium. The 48h treatment is represented in black bars and the 72h treatment is represented in gray bars. 50% inhibitory concentration values (IC50) were calculated based on Presto Blues assays (triplicate assays). N/R, not reported.

Note: n=1 experiment (triplicates are missing).

This reduction in cell viability is possibly related to both an increase in the apoptosis as well as cell cycle arrest, as demonstrated in Figure 2 C and 3 C, respectively, although

there might be other off target effects of this combination which can concur to this cell viability reduction.

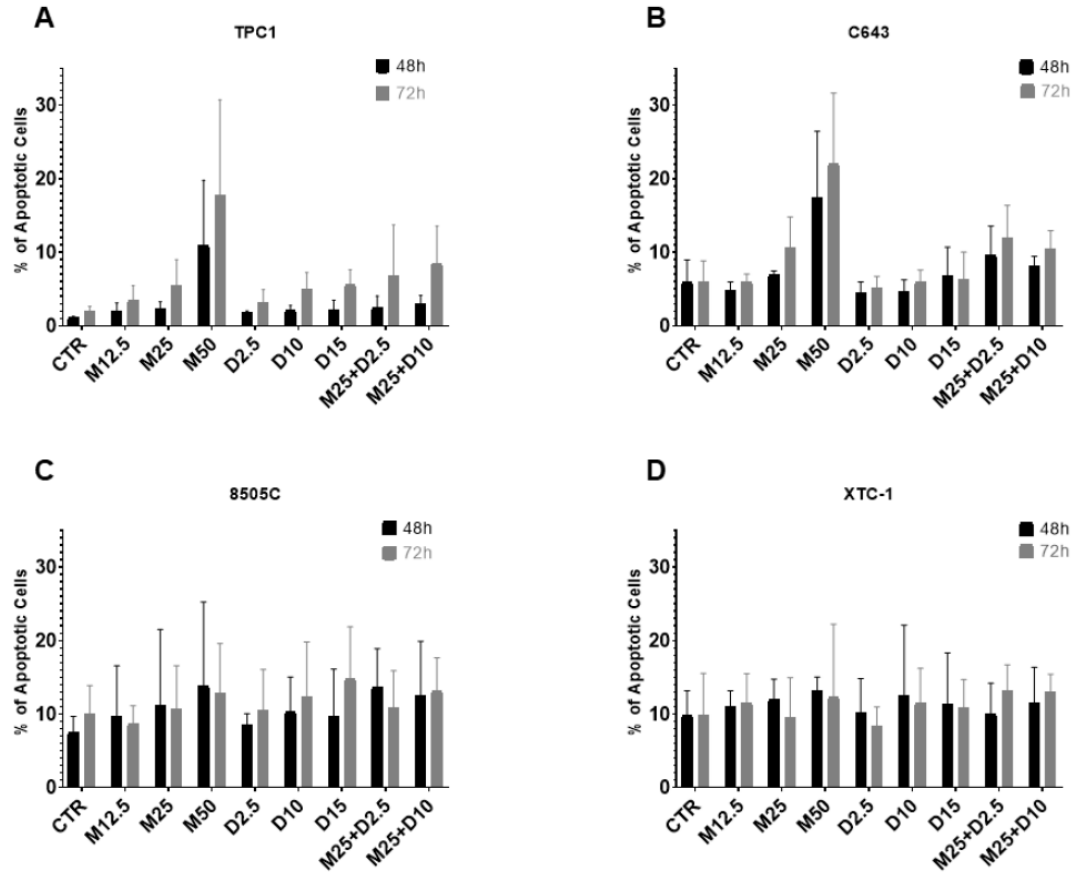


Figure 2. Effects of treatment with Mdivi-1, dabrafenib and Mdivi-1 and dabrafenib combinations in thyroid cell lines apoptosis.

Graphic representation, through a bar chart, of the percentage of TPC1 (A), C643 (B), 8505C (C) and XTC-1 (D) apoptotic cells after treatment with Mdivi-1 (represented as M) – 12.5 μM, 25 μM and 50 μM -, with Dabrafenib (represented as D) – 2.5 μM, 10 μM and 15 μM – and with Mdivi-1 and Dabrafenib combinations – Mdivi-1 25 μM plus Dabrafenib 2.5 μM and Mdivi-1 25 μM plus Dabrafenib 10 μM, during 48h and 72h, determined by Annexin V/PI staining and analysis by flow cytometry. The data are presented as mean ± SD.

Mdivi-1 slightly increases the number of cells in G1 and decreases the number of cells in S phase (Figure 3, A and B). Dabrafenib induces the same effect, but to a greater extent, and it also decreases the number of cells in G2, especially in higher doses (Figure 3 C). Mdivi-1 in combination with dabrafenib does not seem to induce cumulative effects

in cell division. Furthermore, the cell viability does not seem to decrease in a time-dependent manner, since there are only slight differences between the same drug concentration at 48h and 72h post-treatment (data not shown).

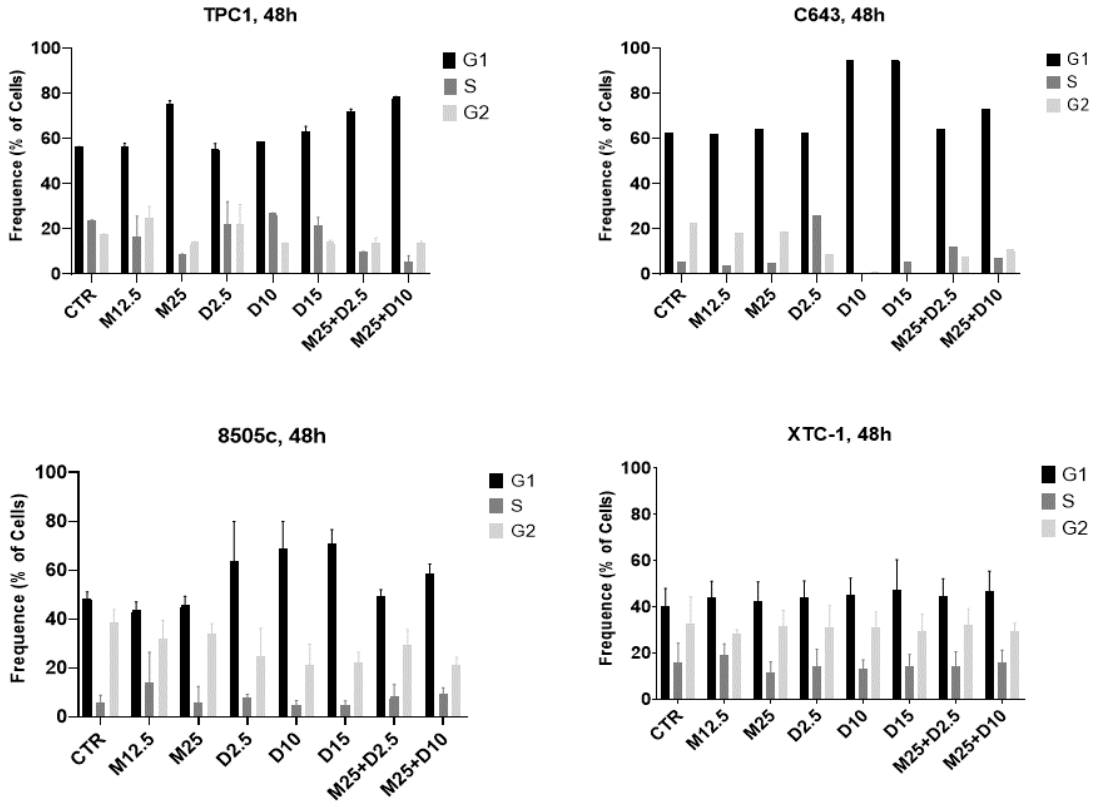


Figure 3. Effects of treatment with Mdivi-1, dabrafenib and Mdivi-1 and dabrafenib combinations in thyroid cell lines cell cycle (48h treatment).

Graphic representation, through a bar chart, of the percentage cells in G1 (A), S phase (B) or G2 (C) after treatment with Mdivi-1 (represented as M) – 12.5 μM, 25 μM and 50 μM -, with Dabrafenib (represented as D) – 2.5 μM, 10 μM and 15 μM – and with Mdivi-1 and dabrafenib combinations – Mdivi-1 25 μM plus dabrafenib 2.5 μM and Mdivi-1 25 μM plus dabrafenib 10 μM -, during 48h. Cell cycle was determined by DNA PI staining and analysis by flow cytometry. Black, dark gray, middle gray and light gray represent TPC1, C643, 8505C and XTC-1 cell lines, respectively. The data are presented as mean ± SD where triplicates were performed.

When we combined Mdivi-1 25 μM with dabrafenib 2.5 μM in the 8505C line, the expression of both total DRP1 and p-ERK (phosphor-ERK) were reduced when compared to the control (Figure 4 and 5).

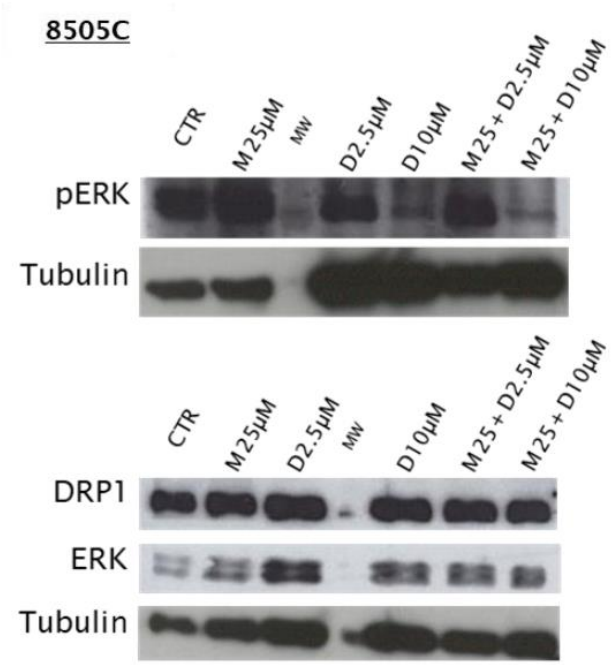


Figure 4. Effects of treatment with Mdivi-1, dabrafenib and Mdivi-1 and dabrafenib combinations in 8505C cell line protein expression.

Western Blot analysis for protein expression of DRP1, phospho-ERK and ERK relative to loading control tubulin in 8505C cell line after 48h treatment. For pERK and the respective tubulin, the molecular weight marker (MW) places in the 3rd position. For DRP1, ERK and the respective tubulin, the molecular weight marker places in the 4th position.

Note: n=1 experiment (triplicates are missing).

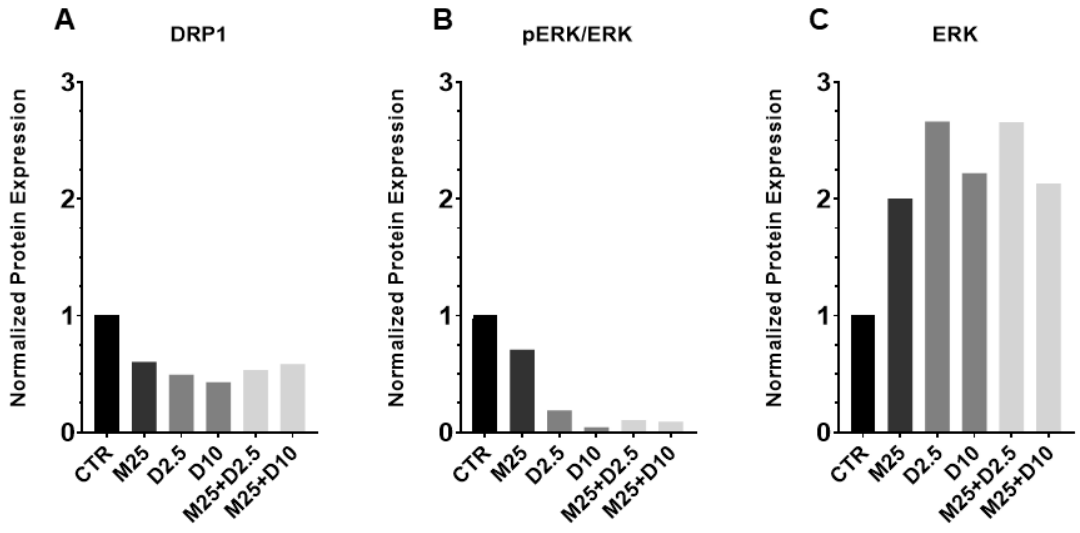


Figure 5. Effects of treatment with Mdivi-1, dabrafenib and Mdivi-1 and dabrafenib combinations in 8505C cell line protein expression.

Graphic representation, through a bar chart, of the protein expression DRP1(A), phosphorylated ERK as counting part of ERK expression (B) and ERK (C) in 8505C cells after treatment with Mdivi-1 (represented as M), – 12.5 μM, 25 μM and 50 μM - with dabrafenib (represented as D) – 2.5 μM, 10 μM and 15 μM – and with Mdivi-1 and dabrafenib combinations – Mdivi-1 25 μM plus dabrafenib 2.5 μM and Mdivi-1 25 μM plus dabrafenib 10 μM -, during 48h, determined by Western Blot analysis. Control is represented in black bars, Mdivi-1 treatments are represented in dark gray bars, dabrafenib treatments are represented in middle gray bars and combination treatments are represented in light gray bars.

Note: n=1 experiment (triplicates are missing).

The same therapeutic approach could be defendable for patients with a RAS-mutated tumor, either PTC (typically FVPTC) or FTC. Although we have not assessed RAS mutations in our IHC series, we anticipate that there would be a benefit of combining Mdivi-1 with dabrafenib. Our assumption stems from the results of cell viability, apoptosis and cell cycle in C646 cell line, which harbors a *H-RAS* mutation. Mdivi-1 reduces cell viability and increases apoptosis in these cells, particularly in its highest 50 μM dose. Dabrafenib seems to induce a cell cycle arrest in this cell line, more than does Mdivi-1. Given the clinical results observed with selumetinib in patients with iodine-refractory TC,

where all 5 patients with *N-RAS* mutations had an increase in iodine update (40), one could postulate a treatment strategy where dabrafenib (or a MEK-inhibitor such as selumetinib or the recently FDA approved trametinib) would be combined with Mdivi-1. Interestingly, after treatment with Mdivi-1 or dabrafenib alone, the C643 cell line does not show differences in the expression of total DRP1 and ERK or p-ERK, although the combination of both compounds seems to produce some effect at this level, which provides additional mechanistic basis for the combination of both treatments (Figure 6 and 7).

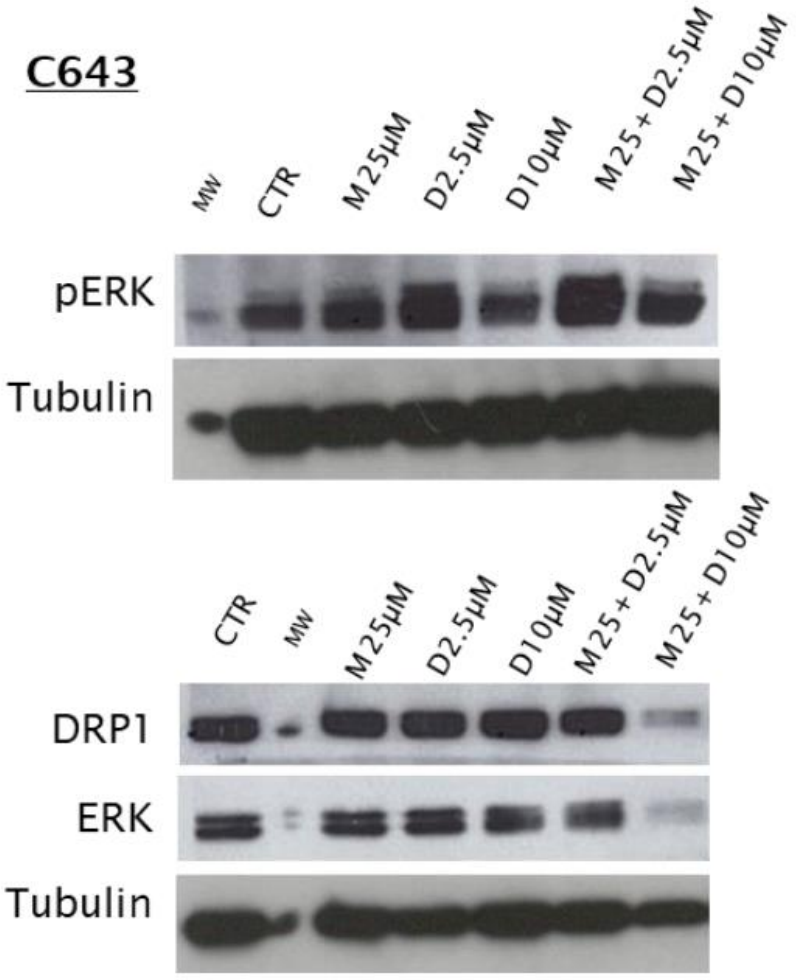


Figure 6. Effects of treatment with Mdivi-1, Dabrafenib and Mdivi-1 and Dabrafenib combinations in C643 cell line protein expression.

Western Blot analysis for protein expression of DRP1, phospho-ERK and ERK relative to loading control tubulin in C643 cell line after 48h treatment. For pERK and the respective tubulin, the molecular weight marker (MW) places in the 1st position. For DRP1, ERK and the

respective tubulin, the molecular weight marker places in the 2nd position. Note: n=1 experiment (triplicates are missing).

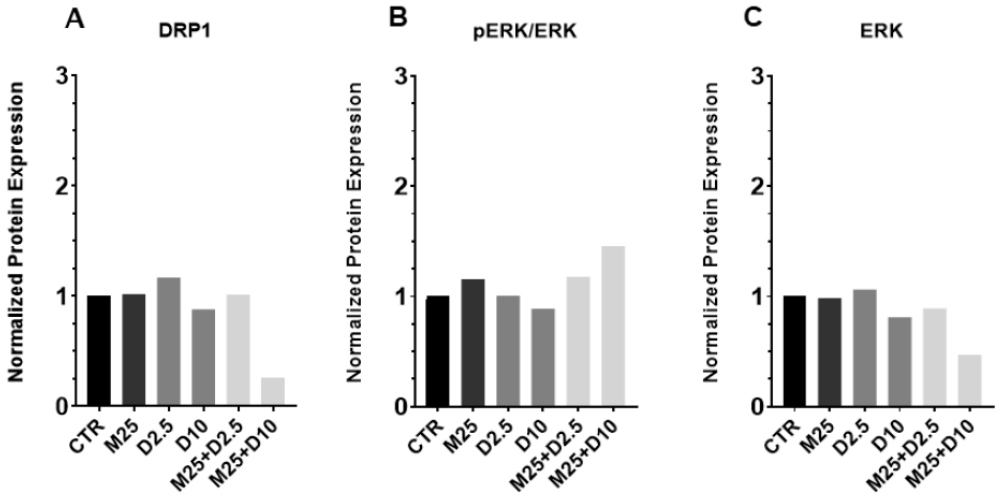


Figure 7. Effects of treatment with Mdivi-1, dabrafenib and Mdivi-1 and dabrafenib combinations in C643 cell line protein expression.

Graphic representation, through a bar chart, of the protein expression DRP1(A), phosphorylated ERK as counting part of ERK expression (B) and ERK (C) in C643 cells after treatment with Mdivi-1 (represented as M), – 12.5 μM, 25 μM and 50 μM -with dabrafenib (represented as D) – 2.5 μM, 10 μM and 15 μM – and with Mdivi-1 and dabrafenib combinations – Mdivi-1 25 μM plus Dabrafenib 2.5 μM and Mdivi-1 25 μM plus dabrafenib 10 μM -, during 48h, determined by Western Blot analysis. Control is represented in black bars, Mdivi-1 treatments are represented in dark gray bars, dabrafenib treatments are represented in middle gray bars and combination treatments are represented in light gray bars. Note: n=1 experiment (triplicates are missing).

To further elaborate on the potential treatment strategy for the oncocytic variants of PTC and HCC (former oncocytic variant of FTC), one needs to take into consideration the genetic and molecular characteristics of these tumors. These characteristics are typically aligned with those of the hystotype they derive from, although it is now recognized that HCC is not a variant of FTC, but rather a new entity on its own. It is important to consider that, although the most recently revised 4th edition of the “WHO Classification of Tumors of Endocrine Organs” defines that oncocytic thyroid neoplasms

with follicular architecture, and no typical nuclei of papillary carcinoma, are now included in a separate group of the Hürthle cell neoplasms, questions exist about the rationale for this change. We have recently revised what is known about the oncocytic thyroid neoplasms, and have raised some of those questions (92). One of the most intriguing results of our IHC data is that all 28 cases of oncocytic variants tested for S616-p-DRP1, including 10 cases of HCC, were negative for S616-p-DRP1 using our IRS score definition. Interestingly, the few cases of renal oncocytoma which we tested for S616-p-DRP1 were also negative for this protein (data not shown). This molecular pattern further supports the concept of a common etiopathogenesis for oncocytic tumors already described previously by our group, and challenges the concept of isolating HCC from all other oncocytic malignant TC (18, 92, 157). As it is shown by the cell viability, apoptosis and cell cycle assays, XTC-1 cell line seems to be the least sensitive to both Mdivi-1 and dabrafenib (Figures 1D, 2D ND 3). Unlike what is seen in the other tested cell lines, Mdivi-1 in combination with dabrafenib does not seem to be effective in decreasing cell viability, with the combination treatment showing less effects than Mdivi-1 or dabrafenib alone. (Figure 1 D). Additionally, there is no difference in the apoptosis at 48h or 72 h for both compounds in this cell line (Figure 2 D). Finally, in XTC-1 cell line Mdivi-1 increases the protein expression levels, whereas dabrafenib decreases it (Figures 8 and 9). Once more, the combined treatment does not seem to have a higher effect on protein inhibition.

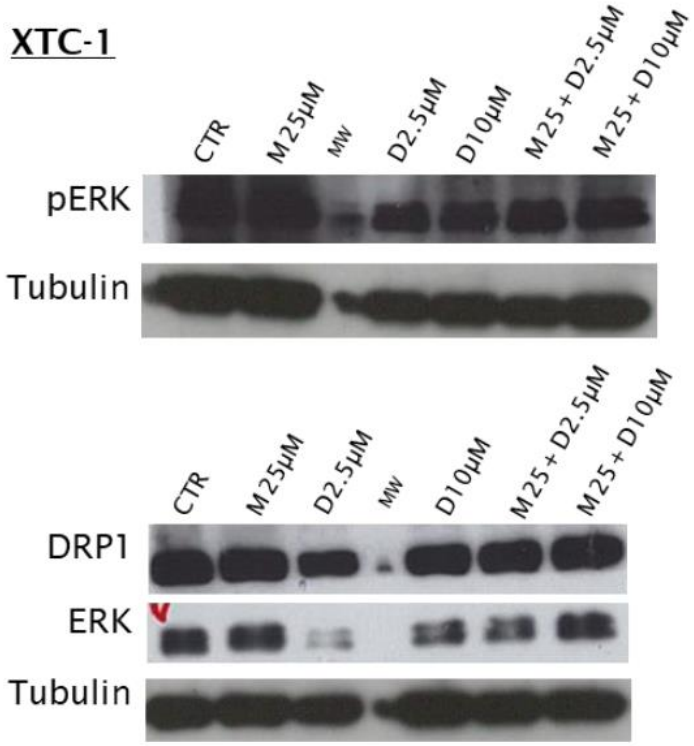


Figure 8. Effects of treatment with Mdivi-1, dabrafenib and Mdivi-1 and dabrafenib combinations in XTC-1 cell line protein expression.

Western Blot analysis for protein expression of DRP1, phospho-ERK and ERK relative to loading control tubulin in XTC-1 cell line after 48h treatment. For pERK and the respective tubulin, the molecular weight marker (MW) places in the 3rd position. For DRP1, ERK and the respective tubulin, the molecular weight marker places in the 4th position.

Note: n=1 experiment (triplicates are missing).

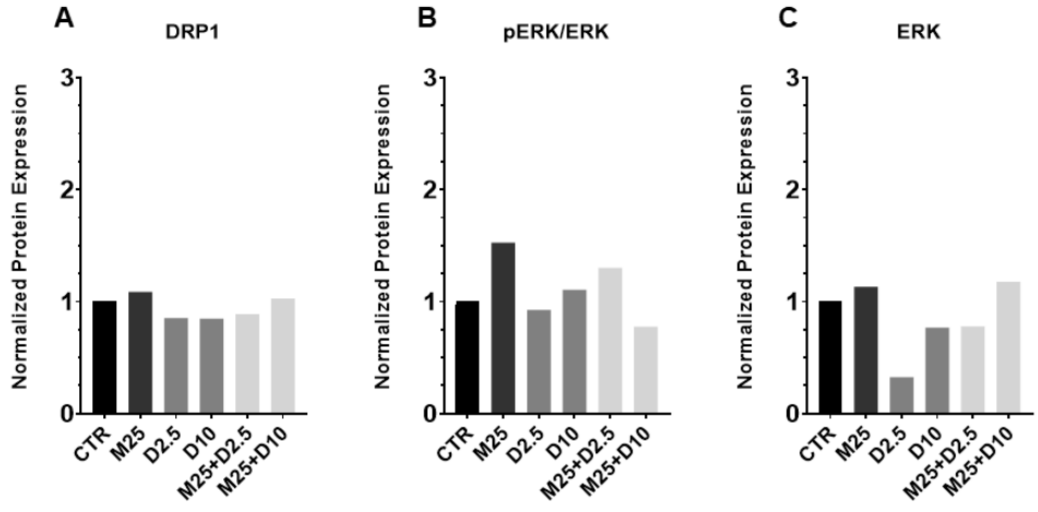


Figure 9. Effects of treatment with Mdivi-1, dabrafenib and Mdivi-1 and dabrafenib combinations in XTC-1 cell line protein expression.

Graphic representation, through a bar chart, of the protein expression of DRP1(A), phosphorylated ERK as counting part of ERK expression (B) and ERK (C) in XTC-1 cells after treatment with Mdivi-1 (represented as M), – 12.5 μM, 25 μM and 50 μM - with dabrafenib (represented as D) – 2.5 μM, 10 μM and 15 μM – and with Mdivi-1 and dabrafenib combinations – Mdivi-1 25 μM plus Dabrafenib 2.5 μM and Mdivi-1 25 μM plus Dabrafenib 10 μM -, during 48h, determined by Western Blot analysis. Control is represented in black bars, Mdivi-1 treatments are represented in dark gray bars, dabrafenib treatments are represented in middle gray bars and combination treatments are represented in light gray bars.

Note: n=1 experiment (triplicates are missing).

The interpretation of these results focusing on a translational treatment application can only be hypothesized. A few characteristics are historically recognized in malignant Hürthle cell tumors (HCT) when compared with other FCDTC (the designation of HCC was used until recently to describe the oncocytic variants of both PTC and FTC): they tend to be diagnosed in older patients, they are associated with more lymph node metastases and they are more frequently associated with RAI resistance, due to a reduced iodine trapping (158, 159). From a cellular perspective, there are at least two important altered processes in these tumors which may correlate with the described clinical phenotype (160) – the apparent low responsiveness or sensitiveness to apoptotic stimuli and the inability to trap iodine (18, 92, 159, 161-164). These are two indissociable aspects that must be tackled to achieve clinical responses in these tumors.

It was already demonstrated by our group that, unlike the other cell lines tested, XTC-1 preserves eight thyroid-specific genes' expression – *PAX8*, thyroid stimulating hormone receptor gene (*TSHr*), thyroid transcription factor 1 gene (*TTF-1*), thyroid peroxidase gene (*TPO*) and *Tg* gene, supporting its more differentiated phenotype (113). Indeed, we have assessed TSHr and NIS mRNA levels in all cell lines, but only detected its expression in the XTC-1 cell line (Figure 10). Interestingly, both Mdivi-1 and

dabrafenib decrease the TSHr mRNA expression, with a higher effect when both compounds are combined (Figure 10). However, only dabrafenib increased NIS mRNA expression showing an effect at 10 mg dose (Figure 11), which is aligned with the clinical activity of this compound in iodine-refractory *BRAF*^{V600E}-mutated patients (39).

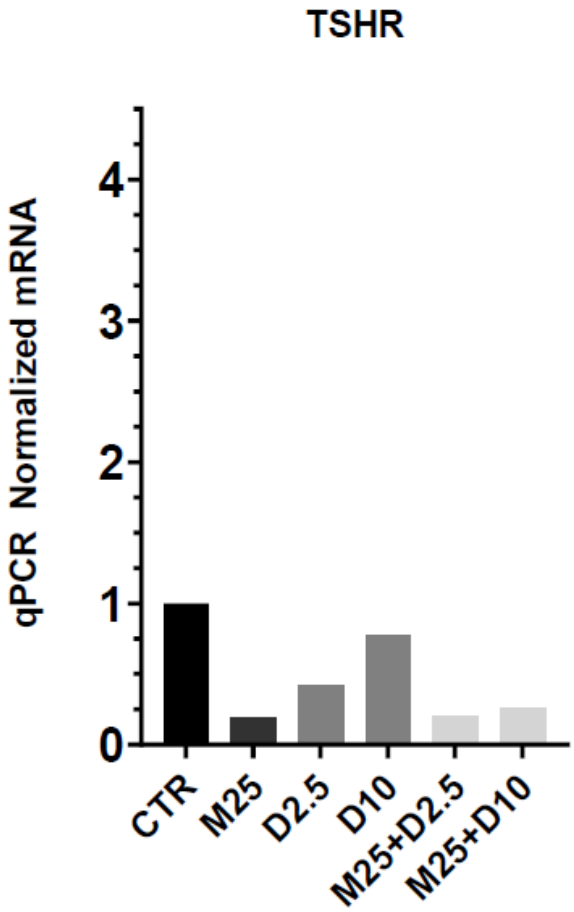


Figure 10. Effects of treatment with Mdivi-1, dabrafenib and Mdivi-1 and dabrafenib combinations in XTC-1 cell lines TSHr mRNA expression.

Graphic representation, through a bar chart, of TSHr mRNA level in XTC-1 cells after treatment with Mdivi-1 (represented as M) – 25 μM -, with dabrafenib (represented as D) – 2.5 μM and 10 μM – and with Mdivi-1 and dabrafenib combinations – Mdivi-1 25 μM plus Dabrafenib 2.5 μM and Mdivi-1 25 μM plus dabrafenib 10 μM -, during 72h, determined by qPCR analysis. The control is represented in black bars, the Mdivi-1 treatment is represented in dark gray bars, the dabrafenib treatments are represented in middle gray bars and the combination treatments are represented in light gray bars.

Note: n=1 experiment (triplicates are missing).

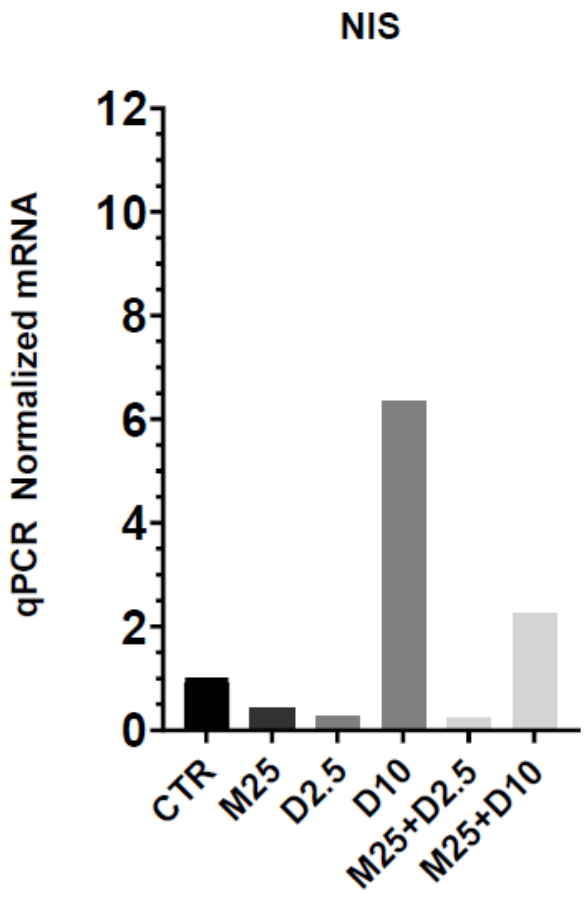


Figure 11. Effects of treatment with Mdivi-1, dabrafenib and Mdivi-1 and dabrafenib combinations in XTC-1 cell lines in NIS mRNA expression.

Graphic representation, through a bar chart, of NIS mRNA level in XTC-1 cells after treatment with Mdivi-1 (represented as M) – 25 μM -, with dabrafenib (represented as D) – 2.5 μM and 10 μM – and with Mdivi-1 and dabrafenib combinations – Mdivi-1 25 μM plus Dabrafenib 2.5 μM and Mdivi-1 25 μM plus Dabrafenib 10 μM -, during 48h, determined by qPCR analysis. The control (DMSO) is represented in black bars, the Mdivi-1 treatment is represented in dark gray bars, the dabrafenib treatments are represented in middle gray bars and the combination treatments are represented in light gray bars.

Note: n=1 experiment (triplicates are missing).

These are encouraging results, knowing that our *in vitro* tumor model XTC-1 does not harbor any *BRAF* mutation, which would predictably respond to its downstream pathway inhibition. However, perhaps the key to unlocking the oncocytic physiopathology is more related with reopening the gate of programmed cell death rather than re-differentiating it. This does not mean that any altered genomic and molecular background is not a target for inhibition, as it should be in the case of *BRAF* por *RAS*-mutated oncocytic TC. This means that we also need to address cell death pathway in ways where we know it is deficient. It has been described that oncocytic tumors frequently harbor mutations in genes encoding OXPHOS complexes (161, 165-167), with an impact on mitochondrial metabolism. Indeed, many of these tumors have a deficient ATP production (114) and an increase of glycolysis (168-172).

The apparent paradoxical de-differentiation of Mdivi-1 when reducing TSHr and NIS expression in XTC-1 cell line, which is known to be defective in OXPHOS, deserves deeper thought. If Mdivi-1 acts as a reversible complex I inhibitor, as already proposed (147), perhaps it further depletes cells from ATP and this, by-itself, further aggravates the tumorigenesis process. If Mdivi-1 reduces mitochondrial biogenesis process, even if by some indirect off-target effect, the benefit of such effect in a full blown oncocytic tumor is likely not useful anymore. It is possible that, by the time we have a clinical diagnosis of an oncocytic carcinoma, the homoplasmy level of an oncogenic mutation and its downstream effects are already critically high and difficult to be reverted. Promoting mitochondrial fusion, may prove to be more effective, while taking advantage of the yet available pool of healthy mitochondria. It would also be interesting to test this strategy in combination with other approaches, such as the use of a glycolysis inhibitor, given the highly glycolytic profile of HCC. These are treatment strategies that should be tested in the future.

Table 4 Theoretical treatment strategies for patients with iodine-refractory FCDTC.

	Non-*BRAF*^{V600E}	*BRAF*^{V600E}	Non-*RAS*	*RAS*
Papillary thyroid carcinoma (PTC)	**Sorafenib or lenvatinib** **+ DRP1 inhibitor (e.g. Mdivi-1)**	**Dabrafenib +/- MEK inhibitor (e.g. selumetinib, trametinib)** **+ DRP1 inhibitor (e.g. Mdivi-1)**		
Follicular thyroid carcinoma (FTC)			**Sorafenib or lenvatinib** **+ DRP1 inhibitor (e.g. Mdivi-1)**	**Selumetinib +** **DRP1 inhibitor (e.g. Mdivi-1)**
Oncocytic variants of PTC (ovPTC) **Hürthle cell carcinoma (HCC)**	**Dabrafenib +** **Mitochondrial fusion promoter**			

7. General discussion and considerations

The optimization of molecular markers with diagnosis, prognosis, and therapeutic objectives in DTC is one of the key priorities of future research, as defined in the most recent ATA 2015 guidelines (23). We have proposed to explore the field of prognosis and treatment, following previous work published by our group indicating that DRP1 is overexpressed in oncocytic neoplasms, particularly in oncocytic TC (112). The hypothesis that DRP1 could have clinical implications in the management of DTC, including in prognosis and treatment, was further supported by various reports published in different tumor models, and in particular in *BRAF*-mutated melanoma. In the latter, S616-p-DRP1 expression dichotomized $BRAF^{WT}$ from $BRAF^{V600E}$ melanoma, which suggested a mechanistic link between S616-p-DRP1 and $BRAF^{V600E}$ activation in melanoma (136). Given the fact that approximately half of PTCs harbor this oncogenic mutation, we questioned whether a similar mechanism would be present in PTC. Besides melanoma, a high expression or enhanced activation of DRP1 have been associated with malignant phenotype in lung cancer (139, 173), breast cancer (135, 137), endometrial cancer (174), ovarian cancer (175), glioblastoma (142, 176), colorectal cancer (177), pancreatic cancer (154), liver cancer (146, 178) and mesothelioma (179). Despite this, a recent report of a relatively small number of human tumor samples has suggested that DRP1 is downregulated in some malignant tumors as compared with normal tissue, which makes the likely contribution of DRP1 to cell malignant transformation and/or cancer progression far from being a simple process (180). Although counterintuitive, this is not surprising, given the role of DRP1 in countering cell processes, which we tried to summarize in our review paper (119). The attempt to explore DRP1 as a prognostic factor has not yielded results in lung cancer yet, suggesting a worse overall survival in patients with high DRP1 expression based on Oncomine and TCGA databases, although with some inconsistencies in the data (181).

Again, these inconsistencies are possibly explained by the pleotropic role of DRP1, and no simple theory can explain the role of DRP1 in tumorigenesis and cancer progression. These different roles are likely dependent on the genetic, environmental and tissue specificities, which may also explain why DRP1 may have different biological implications in different tumor models.

This brings us back to our project, and the need to characterize the expression pattern of DRP1 in a large series of FCDTC to conclude on the potential role as a prognostic biomarker. We have initially started with the IHC assay for total DRP1, however later in the project we have decided to pilot S616-p-DRP1 antibody in a sub-sample of 98 tumor tissue samples of our series, anticipating that this active form would have a higher functional correlation with the tumorigenesis process. Based on our IHC results, we found that DRP1 expression was positive in 65.3% of the cases, whereas S616-p-DRP1 expression was positive in only 17.3% of the cases. From a theoretical perspective, if we would be searching for the ideal candidate which is able to predict poor clinical outcomes or aggressive disease in DTC - knowing that patients whose disease will likely become RAI-refractory accounts for about 15% of the overall patients -, S616-p-DRP1 would be closer to that prototype.

It seems clear from our work that DRP1 is more highly expressed in PTC hystotypes, particularly in classic variants of PTC. When we compared PTC (excluding all “aggressive variants”) in combination with FVPTC versus FTC, no significant difference in DRP1 expression was found. However, when we compared PTC (excluding all “aggressive variants”) with FVPTC and FTC combined, a significant difference in DRP1 expression was found, with 73.8% of the PTC cases staining positive for DRP1 versus 56.9% of the follicular tumors. This supports the idea that FVPTC are probably more similar to FTC than to PTC, beyond differences in morphology and genetic identity (115). We also found that the aggressive variants of PTC, combined with PDTC (n=24),

had a significantly lower DRP1-positive expression, although we acknowledge that the number of those cases in our series is small. However, we have not found these differences when we tested the same comparisons for S616-p-DRP1. This may be explained by the fact that the 98 cases tested for the active protein represented a too small sample to explore potential differences between the main hystotypes and their variants. DRP1 is known to be involved in various cellular processes, including cell cycle progression, cell death, and differentiation (177, 182-184). It is tempting to hypothesize that tumors with lower DRP1 expression might reflect the phenotypic expression of less differentiated tumors and/or tumors presenting a different genetic mutational background. FVPTC and FTC are differentiated tumors and this, *per se*, would not explain the difference we observed in DRP1 expression pattern. We are also not able to suggest whether a particular genetic background, such as *RAS* mutation or even *TP53*, may partially explain these differences.

Aligned with reports in other tumor models, the higher proportion of positive DRP1 expression in our series was associated with invasive traits of the tumor, as supported by a significantly higher number of positive cases in tumors with thyroid capsule invasion. However, this invasive pattern does not seem to have any clinical implication at the level of a higher rate of lymph node metastases, and more importantly, distant metastases. This is not unexpected, since local and distant metastases seem to have different molecular signatures in FCDTC (185). DRP1 has been implicated in invasive characteristics of tumor cells *in vitro* in thyroid, breast and lung cancer (137, 138, 173, 186) and *ex vivo*, in breast cancer (137). The inhibition of DRP1 was shown to inhibit the invasive and migration characteristics of those cells (137, 138, 173, 186). The mechanisms by which DRP1 is associated with the invasive patterned tumors are still under exploration, however it has already been shown that hypoxia induces the *in vitro* upregulation of DRP1 in glioblastoma, with concomitant mitochondrial fission and cell

migration (142). This link with hypoxia was also found in a breast cancer metastatic cell line, an effect that was reverted by inhibition of DRP1 (138).

At variance with DRP1, its active form S616-p-DRP1 presents a stronger association with cancer invasion. A higher proportion of S616-p-DRP1-positive cases was significantly associated with infiltrative margins, thyroid capsule invasion, and lymph node invasion, which is aligned with previous reports on the invasiveness role of S616-p-DRP1 in malignant tumors, such as breast and lung cancer (137, 173). One might anticipate that, given the significant association with lymph node metastization, an association might also be seen with the PTC hystotype. However, this was not observed, which further supports the thesis that S616-p-DRP1 may be a stronger predictor of locally invasive disease, irrespective of hystotype.

One of the most unexpected results of our study was the fact that S616-p-DRP1-positive cases were significantly lower in the 28 cases of oncocytic neoplasms. In fact, all cases were negative for S616-p-DRP1. This included 11 cases of HCC, as per the most recent WHO classification (16). We would expect that, given the higher mitochondrial biogenesis previously described in these tumors, S616-p-DRP1 would be highly expressed, since it is required for the process of mitochondrial fission preceding mitophagy (187). Our group has shown that DRP1 is overexpressed in oncocytic thyroid neoplasms, particularly in oncocytic carcinoma, suggesting that mitochondrial dynamics are dysregulated in Hürthle cells (186). Hürthle cell tumors are defined as tumors composed of more than 75% of cells characterized by the cytoplasmic accumulation of abundant mitochondria that frequently display abnormal morphology (18). It has been suggested that these tumors have a deficient mitophagy process, either as a consequence of electron chain complex mutations and/or as a response to oxidative and environment insults (188). These mechanisms are the basis for the hypothesis of an imbalance between mitochondrial biogenesis and mitochondrial turn-over explaining the phenotype of these tumors (18, 157, 165, 167, 189-192). Based on our previous work,

we had suggested that mitophagy mediated by DRP1 would be a determinant process in the oncocytic phenotype, allowing mitochondrial accumulation (186). The fact that S616-p-DRP1 expression was negative in oncocytic tumors does not oppose to this concept. One could hypothesize that this sort of self-regulatory mechanism, where mitochondrial biogenesis inexorably supplants a defective mitochondrial turnover, would increase DRP1 demand, both on its activation, through phosphorylation, and also on the ubiquitination process preceding its degradation. This, in its turn, could lead to a retrograde signal restraining the biogenesis stimulus. If this hypothesis proved to be true, a high level of S616-p-DRP1 is likely expressed in the early stages of oncocytic transformation, but once the tumor reaches the established oncocytic phenotype, the opposite phenomenon is observed. If such a feedback loop exists, it probably exerts its effects at the post-translational level and not in the nuclear genome, since we found no significant difference between total DRP1 expression in oncocytic versus non-oncocytic tumors.

Mitochondrial fission is a complex process, which is dependent on the right amount and proper functioning of other dynamin-related proteins, post-translational modifications, and also on the mitochondria lipid cardiolipin. It is therefore expected that the ultrastructurally defective oncocytic mitochondria in an established tumor may lack the molecularly fit machinery needed for DRP1 oligomers to assemble in bigger helical-like structures, as described recently (193). This hypothesis may further build on the theory of a defective mitophagy supporting a vicious circle in oncocytic transformation.

The lower S616-p-DRP1 expression observed in tumors with oncocytic morphology could explain, at least partially, why these tumor cells are less prone to cell death. We know that in healthy normal cells, BAX oligomerizes in the same microdomains of the OMM, alongside with DRP1 and MFN2, before apoptosis (194). It is reasonable to admit that this process is altered either by the absence of a structurally fit OMM and/or by the energetic imbalance and ATP deficit secondary to a compromised OXPHOS process. Another concurrent mechanism may be the lack of a functional

PARKIN protein which is unable to locate in the OMM due to a mutation in *PARK2* gene, as already described in HCT and in the XTC-1 oncocytic cell line (195), and/or due the reduction of membrane potential associated with the defective OXPHOS. Indeed, it has been described that PARKIN accumulation in mitochondria is dependent on voltage more than on ATP or pH levels (196). Most of the fusion and fission proteins are a substrate of PARKIN as part of their ubiquitination and subsequent mitophagy, and the fact that this process is compromised in HCT does not allow a proper quality control of the defective mitochondria pool. Mitochondrial outer membrane permeabilization (MOMP), with release of apoptotic factors and activation of the apoptosis pathway(s), is also dependent on the appropriate balance of properly functioning activated fission and fusion proteins. Whether the lack of response to cell death stimuli also explains RAI resistance it-self in these tumors, directly or indirectly, is a question that deserves further study.

Still on the topic of oncocytic transformation and tumorigenesis, the most interesting result of our mechanistic work derived from the XTC-1 cell line. This oncocytic cell line, the only of the four tested cell lines expressing baseline levels of NIS and TSHr, reverted this expression pattern by the action of Mdivi-1. XTC-1 derives from a HCC breast metastasis, and harbors a mutation in the ubiquinone oxireductase chain 1 (ND1) gene (complex I) and in cytochrome B (CytB) gene (complex III) (189). As mentioned already, XTC-1 was also shown to harbor a mutation in the Parkinson protein 2 E3 ubiquitin protein ligase gene (*PARK2*). Therefore, we would assume that this cell line might represent a valuable *in vitro* model to explore the mechanisms behind resistance to apoptosis and RAI therapy observed in HCC. The fact that these cells are differentiated to the point of expressing thyroid specific genes transcripts, but lose this differentiation pattern upon treatment with Mdivi-1, suggests that the inhibition of DRP1 in HCC is causing a harmful effect, perhaps because it inhibits the remaining available pool of healthy, functioning mitochondria. Through its activity on DRP1 and/or other unknown off-target effects, Mdivi-1 is potentially increasing the energetic and metabolic

stress levels to a threshold where the cells are forced to gain some sort of stemness traits to be able to survive. We know that the inhibition of DRP1-mediated mitochondrial fission decreases the oxygen consumption rate and causes metabolic stress in brain tumor initiating cells (BTICs) (176), and it is recognized that lactate and ketone use by cancer cells promotes a "cancer stem cell" phenotype, with a correlation with poor outcomes in breast cancer (197, 198). This evidence supports glycolytic metabolism as a driver of stemness characteristics. If this phenomenon occurs in XCT-1 cells as a result of Mdivi-1 action is a hypothesis that would need to be tested, for example, by assessing the expression of stemness markers such as octamer-binding factor 4 (OCT4). It would also be interesting to test how mitochondria shape and metabolism is changed after Mdivi-1 treatment and compare this with the effects of a mitochondrial fusion promoter. In a way, we could compare the oncocytic tumor mitochondria disfunction model with the one of heart ischemia-reperfusion injury, where mitochondrial fusion promoters could potentially have some benefit. Given the effect of some MAPK inhibitors in the redifferentiation of RAI-resistant tumors, we would propose to test a dual strategy based on a combination of a MAPK inhibitor with a mitochondrial fusion promoter. In our XTC-1 cell line model, dabrafenib increased the levels of NIS and TSHr expression. Interestingly, selumetinib did not prove to increase NIS expression in TPC-1 or XTC-1 cell line (data not shown). For this reason, we would favor the use of dabrafenib, particularly in the case of a *BRAF*^{V600E}-mutated tumors.

We acknowledge the questions recently raised on the mechanism of action of Mdivi-1, casting a doubt about its mechanism of action as a GTPase inhibitor and defending it as a reversible mitochondrial respiration complex I inhibitor (147). This compound has been used as a putative DRP1 inhibitor, and was shown to inhibit mitochondrial division in yeast and mammalian cells, delay apoptosis by inhibiting OMM permeabilization, and block BAX/BAK-dependent cytochrome c release from mitochondria (132). It has also been used as a neural protector in neurodegenerative

and in ischemia-reperfusion injury disease models (130, 131, 199). While it is not confirmed that Mdivi-1 acts mainly through a GTPase inhibition, it is recognized that Mdivi-1 recapitulates the morphological effects of DRP1 small interfering RNA or K38A dominant-negative DRP1 (200). In HeLa cells, down-regulation of DRP1 inhibited cell growth, caused loss of mtDNA and uncoupling of the electron transport chain (ETC), decreased cellular respiration and increased reactive oxygen species (ROS) levels (201). In most of the published mechanistic studies where Mdivi-1 was used as an anti-cancer agent, it has consistently induced mitochondrial morphology changes, as well as metabolic, anti-proliferative, pro-apoptotic and anti-invasive effects. It was also recognized that Mdivi-1 may have off-target effects. As an example, Mdivi-1 induced replication stress, mitochondrial dysfunction and cell apoptosis in a DRP1 independent fashion, in multidrug resistant breast cancer cells (140). Despite this, the consistent effects on different tumor type models supports our choice for Mdivi-1 as a mitochondrial modulator. We do recognize that morphological and metabolic studies are lacking in our project. The morphological studies would be very relevant to support the putative mechanism of action as a mitochondrial fission inhibitor and to better interpret the biology behind a unique tumor model, such as HCC. As already stated, mitochondria morphology seems to be a better predictor of response to these agents (179).

The significant association between the lower expression of DRP1 and a higher number of radioiodine treatments may be an observation in favor of a link between higher DRP1 expression/activity and FCDTC differentiation. Interestingly, a statistically significant association between higher S616-p-DRP1-positive expression and a higher mean cumulative radioiodine dosage was observed. It is reasonable to admit that patients with locally invasive disease, including lymph node metastases, may have been treated with higher radioiodine dosages, even if the number of radioiodine treatments has not significantly differed in the overall patient population tested. In this respect, the apparent contradictory results between radioiodine treatment intensity observed for total

DRP1 and S616-p-DRP1 expression could be justified. On a different, although theoretic, perspective, the association between a lower proportion of DRP1-positive tumors and a higher number of RAI treatments could indicate that these tumors harbor the phenotypic expression of a less self-regulated and less differentiated cellular clonal expansion, which could also explain, at least to some extent, why the majority of patients with distant metastases were negative for S616-p-DRP1. However, even though the two clinical events are many times coincident, the biological mechanisms involved in distant metastization possibly differ from the ones related to RAI resistance. It has been reported that DRP1 is involved in the mitochondria dynamics supporting differentiation of embryonic stem cells, oocytes, myocytes and neuronal cell differentiation (202-205), and it is reasonable to accept that the non-active form(s) of DRP1 may be linked to less differentiated cells. The assessment of differentiation markers, such as NIS expression and iodine cell uptake, and their crosstalk with DRP1 as a key effector of mitochondrial bioenergetics and dynamic, would shed a light into the potential mechanisms of RAI resistance. To this point, it would be clinically relevant to study the effect of iodine treatment and TSH suppression on DRP1 under a context of both high and low DRP1 expression. This would eventually help clinicians to further tailor treatment strategies for those patients who have persistent disease, become refractory to iodine treatment, and will eventually die from distant metastization. This would be particularly relevant also in HCC treatment management, which seem to be more resistant to RAI (92).

Contrary to our expectations, we did not find any association between DRP1 or S616-p-DRP1 expression and distant metastases. DRP1 expression and/or activation has been associated with cell migration and invasion in breast cancer, lung cancer, glioblastoma and TC, and the pharmacological or genetic inhibition of DRP1 have been effective in reducing this cell behavior *in vitro* and in mouse models (137, 142, 173, 186). Although the number of patients who presented distant metastases and who were tested for S616-p-DRP1 was low, it is still noteworthy that 14 out of 16 were negative for S616-

p-DRP1. Only a relatively small number of patients will eventually die from the disease, which makes it difficult to relate DRP1 expression with this long-term clinical outcome. Nevertheless, our results showing that the 5 patients who died from TC were S616-p-DRP1-negative seem to be in line with the lower number of DRP1-positive cases in less differentiated tumors.

Unlike what was seen in melanoma, no association was found between DRP1 or S616-p-DRP1 positivity and *BRAF*^{V600E} mutation. There was also no association with *TERT* promoter mutations, which indicates that DRP1 regulation is not dependent from the genetic background in FCDTC. Warburg has once said that "*mutation and carcinogenic agent are not alternatives, but empty words unless metabolically specified*" (206). Perhaps DRP1 role in FCDTC is also metabolically specified, beyond a genetic signature. In epithelial tumors, where proliferation rates are high, DRP1 is possibly a key target to reduce cell proliferation. As shown by Tanwar *et al*, DRP1 co-expresses with cell-cycle module responsible for mitotic transition, and the same was shown to be true for many other tumors (175). In thyroid cells, which are known to rarely proliferate and who are subject to high oxidative stress - and particularly in HCC -, DRP1 expression may have a different pattern. It would be interesting to assess if metabolism and immune system genes are typically co-expressed with DRP1 in TC.

We propose that the positive expression of S616-p-DRP1 can be used as a surrogate marker of infiltrative, locally invasive tumors and lymph node metastases, and hence of a higher probability of persistent disease or disease recurrence in DTC. Under this context, S616-p-DRP1 could be used as a molecular biomarker, in combination with the already established prognostic clinico-pathologic factors, for the purpose of pre- and post-operative risk assessment. On the opposite spectrum of its biological significance, a question is raised about whether S616-p-DRP1 could be used in non-metastatic high-staged disease, as a negative predictor of worse outcomes, in particular distant

metastases spread and disease-related death. We acknowledge that this is a hypothesis that needs further testing, as the number of patients and events which support this suggestion in our IHC study were very small to conclude on any association.

Although the overall prognosis of patients with TC is usually good, with a 10-year survival rate of over 90% (59), up to 15% of the patients will eventually evolve to RAI-resistant disease. The 10-year survival of patients who have RAI-refractory metastatic disease is only 10% (61, 207). This represents up to 15% of the TC patients who still present with an unmet medical need. We believe that mechanistic studies should be undertaken exploring treatment strategies based on modeling DRP1 and MAPK activity in various TC models. The concept of interfering with mitochondrial dynamics as a strategy to suppress the potential metastatic effect of some targeted therapies, or to overcome treatment resistance to others, has already been proposed in other tumor models (152, 154, 208). Whether this represents solid ground for treatment of TC is a field that deserves further investigation. This may pave the way to further tailor treatment strategies for patients who have persistent disease, become refractory to iodine treatment, and will eventually die from distant metastization - where the unmet medical need exists.

8. Limitations

Despite the merit of analyzing a considerable large-sized sample of patients with FCDTC, and the mechanistic work which was developed to further complement a translational application, there are some limitations to this project's endeavor.

First, given the overall good prognosis of TC, with fortunately only a limited number of patients dying due to TC, large numbers of patient would be needed to increase the number of death events, and to reduce the effect of patient and tumor biology heterogeneity, therefore supporting statistically robust data. In particular, correlation with clinically relevant outcomes such as distant metastases, cancer-specific death and overall death were difficult to derive, although we do not exclude that this would be possible to achieve in a bigger patient sample.

Second, our IHC results were achieved on the basis of a retrospective analysis of patients´ clinical data and tumor samples, which may add some potential biases. Some of these may be related to the heterogeneity of surgical procedures, as well as the indication for, and doses of, RAI therapy, which have certainly evolved over the past decades. The use of TKIs, although residual in our series, can also be accounted for as potential bias factor in the last decade. Also, because of the retrospective nature of our study, we have used the 7th edition of the AJCC/IUAC TNM staging system, which although far from being obsolete, is certainly less predictive of patients' outcomes than the most recently used 8th edition (10). The histopathological classification of the tumor cases was done according to the previous WHO edition (15), where HCC was still considered a variant of FTC. These would be factors and variables whose biases we could better control in a prospectively assessed patient population. We also should extend the analysis to further genotypes, including *RAS* and *TP53* mutated tumors.

Additionally, it would be important to increase the representativity of patients with distant metastases, as mentioned before. Nevertheless, none of these limitations had an impact on the results and our conclusions, particularly regarding the association with

invasive disease. We have tested eight different definitions of DRP1- and S616-p-DRP1-positivity based on various IRS and intensity thresholds, and all have showed the same trends herewith reported (data not shown). To this point, the need for the IHC IRS score validation is an objective which could be achieved in a prospective study. Although it became clear that our own definition of positive expression resulted in significant association with clinico-pathological characteristics, which may support further mechanistic exploratory studies, S6161-p-DRP1 expression is likely not binary in tumor tissue. Eventually, further characterization of other IHC testing and assessment methods and algorithms would be interesting to explore, envisioning a future application in clinical practice. Our results should be prospectively validated.

Finally, there are some limitations in the accuracy and validity of our mechanistic cell line assays, in particular due to the need to implement triplicates of the immunoblotting assays in all four cell lines, and also the mRNA expression assays in the XTC-1 cell line. Additionally, it would be very important to ascertain the morphological effect of DRP1 inhibition on mitochondria, as mitochondrial morphology can be a strong predictive of response to DRP1 inhibition (179). The results should also be confirmed through DRP1 genetic inhibition, given the potential off-target effects of Mdivi-1 and yet non-consensual mechanism of action of this putative DRP1 inhibitor (132, 140, 147).

9. Future directions

1. Prognosis

We propose that the positive expression of S616-p-DRP1 can be used as a surrogate marker of infiltrative, locally invasive tumors and potentially lymph node metastases, and hence of a higher probability of persistent disease or disease recurrence in DTC. Under this context, S616-p-DRP1 could be used as a molecular biomarker in combination with the already established prognostic clinico-pathologic factors for the purpose of pre- and post-operative risk assessment. In the pre-operative context, before the critical pathology information about factors, such as tumor margins pattern, capsule and vascular invasion, S616-p-DRP1 positivity pattern could guide the clinician in choosing for a more or less intensive surgical approach (e.g. lobectomy versus thyroidectomy, with or without lymph node dissection). In the post-operative context, the use of S616-p-DRP1 in TC risk assessment may have implications on the decision to complete thyroidectomy in stage I patients who may have a higher risk of disease recurrence, and who were submitted to lobectomy only. Also, it may help decide on the initiation of RAI therapy as an adjuvant therapy following thyroidectomy, the doses of RAI, and the level of TSH suppression, particularly for patients who are considered as having a low risk of disease recurrence as per ATA guidelines. It can also support the need for a more intensive follow-up of these patients. On the opposite spectrum of its biological significance, S616-p-DRP1 could be used in non-metastatic stage II and stage III patients, where it could play a valuable role as a negative predictor of poor outcomes, in particular distant metastases spread and disease-related death, possibly more useful in FTC where most of distant metastases are reported. Indeed, the current staging systems are far from ideal in predicting mortality from TC, and it would be of significant clinical value if we could better predict the risk of death from DTC in an individual patient (23). This would likely tailor therapy and follow-up strategies to address these risks in a more effective way.

2. Treatment

One of the key conclusions of our work is that the importance and biological significance of DRP1 in TC is better characterized by the role and dynamic of its activated and inactivated form(s) than by DRP1 expression it-self. It is difficult to ascertain the role of DRP1 when we are still far from understating the different functions of each of its activated forms in normal and tumoral tissues. This becomes an even more challenging objective when we are using a compound which mechanism of action is not totally characterized.

Exploring treatment opportunities having S616-p-DRP1 as a molecular target is a highly complex task, when compared to unravelling its potential as a molecular prognostic marker. DRP1 does not only participate in cell cycle and cell death processes, but it is also linked to the cell quality control processes, particularly mitophagy, mitochondrial metabolism and cell energetic homeostasis. It would be important to understand how DRP1 dynamics - in concrete the balance between S616-p-DRP1 and S637-p-DRP1, and between fission and fusion - interact with both the metabolic signature and mitophagy process in a given tumor model. Certainly, PTC and FTC are biologically different in this respect. There is a suggestion that the S637-p-DRP1 cytosolic state dominates over S616-p-DRP1 mitochondrial state in HeLa cells, at least in interphase, and this balance is altered in response to increased Ca^{2+} levels and calcineurin activation, with dephosphorylation of S637-p-DRP1 (209). Besides the post-translational modifications of DRP1 that model its location and function, DRP1 also interacts with other mitochondrial fission and fusion proteins, particularly MFN2, MFF and OPA1. Whenever possible, the characterization of the expression of these fission-fusion proteins should be sought.

For both PTC and FTC, we should further explore the effects of targeting DRP1 and MAPK pathway, alone and in combination, as we started doing in this project. Different genetic and metabolic signatures may interact differently with DRP1 in these

two different hystotypes. Also, ovPTC and HCC – the latter previously classified as oncocytic variants of FTC - represent a distinct pathologic model, and both entities are possibly more similar than what we are able to defend (92). We believe that a cybrid model of HCC, using XTC-1-derived mitochondria, may be a good starting point for this analysis: one where we could modulate respiratory (OXPHOS) or glycolytic activities, and mitochondrial dynamics. This could be done using different metabolic, respiratory and fusion/fission inhibitors. Exploring the effects of this modulation on programmed cell death, cell cycle, invasion and migration, and iodine uptake and organification would possibly advance our understating of how metabolism, cell survival and mitochondrial biogenesis are related.

10. Conclusions

We intended to explore the potential of DRP1 as a prognostic and predictive biomarker in DTC. The highest unmet medical need in this respect is two-pronged – first, the identification of new biomarkers that can be strong predictors of poor prognosis and disease recurrence, and second, overcoming resistance to the standard of care therapy which, after surgery, is still RAI. We have contributed to the clarification of the role of DRP1 as a biomarker in DTC, through the definition of S616-p-DRP1 expression as a potential biomarker candidate for stratification of pre- and post-operative risk assessment. We have also made advances in the molecular characterization of oncocytic neoplasms and explored treatment strategies, which can be tested aiming to explore and overcome the mechanisms of disease in these and other DTC.

DRP1 is an attractive molecular target which has gained increasing attention in oncobiology, particularly given its association with proliferative and invasive characteristics of tumor cells and its communication with key signaling pathways, which are often activated in this disease. Patients' access to compounds which are able to revert RAI-refractoriness in TC has recently become a reality, and the definition of how modulation of mitochondrial dynamic and metabolism may synergize with those is a promising field which deserves further mechanistic and *in vivo* studies.

Upon completing the PhD program, we believe we have made important advances in the assessment of risk in DTC and opened new fields for investigation in the areas of prognosis and treatment of TC.

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Appendix I

(published articles)

Lima AR, Santos L, Correia M, Soares P, Sobrinho-Simões M, Melo M, Máximo V. **Dynamin-Related Protein 1 at the Crossroads of Cancer**. Genes (Basel). 2018;9(2).

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genes

MDPI

Review

Dynamin-Related Protein 1 at the Crossroads of Cancer

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Abstract: Mitochondrial dynamics are known to have an important role in so-called age-related diseases, including cancer. Mitochondria is an organelle involved in many key cellular functions and responds to physiologic or stress stimuli by adapting its structure and function. Perhaps the most important structural changes involve mitochondrial dynamics (fission and fusion), which occur in normal cells as well as in cells under dysregulation, such as cancer cells. Dynamin-related protein 1 (DRP1), a member of the dynamin family of guanosine triphosphatases (GTPases), is the key component of mitochondrial fission machinery. Dynamin-related protein 1 is associated with different cell processes such as apoptosis, mitochondrial biogenesis, mitophagy, metabolism, and cell proliferation, differentiation, and transformation. The role of DRP1 in tumorigenesis may seem to be paradoxical, since mitochondrial fission is a key mediator of two very different processes, cellular apoptosis and cell mitosis. Dynamin-related protein 1 has been associated with the development of distinct human cancers, including changes in mitochondrial energetics and cellular metabolism, cell proliferation, and stem cell maintenance, invasion, and promotion of metastases. However, the underlying mechanism for this association is still being explored. Herein, we review the published knowledge on the role of DRP1 in cancer, exploring its interaction with different biological processes in the tumorigenesis context.

Keywords: dynamin-related protein 1; mitochondrial biogenesis; tumorigenesis; cancer; metabolism

1. Introduction

Mitochondrial dynamics is known to have an important role in the so-called age-related diseases, including obesity and type 2 diabetes, Parkinson's disease, Alzheimer's disease (AD), and cancer. Despite this, research on cancer and mitochondrial dynamics has only recently started to be unveiled [1–4].

Mitochondria are organelles involved in many key cellular functions, such as adenosine triphosphate (ATP) production, cell anabolic and catabolic functions, calcium signaling, cell division and differentiation, and cell death [5–7]. Mitochondria respond to physiologic or stress stimuli by

adapting their structure and function, which are intimately connected [8]. In recent years, much has been explored on the key molecules and processes that intervene on, or drive, some of these structural and functional changes. Perhaps the most important of such structural changes is the phenomena of mitochondrial fission and fusion, which occur in normal cells, as well as in cells under dysregulation, such as cancer cells, as reviewed by Chen and Chan, and Westermann [9,10]. Mitochondrial fission secures an adequate number of mitochondria to support growing and dividing cells [8,9]. Mitochondrial fission also generates new organelles and represents a quality control mechanism by eliminating damaged mitochondria through selective autophagy, also called mitophagy [9,11]. Mitochondria fusion, on the other hand, is required for maximal ATP production when mitochondria need to rely on oxidative phosphorylation, or when they have to react to stress stimuli, in which case they appear as elongated *healthy* organelles that complement the dysfunctional mitochondria [12–14]. Fusion also allows the exchange of proteins, metabolites and mitochondrial DNA (mtDNA) within the mitochondrial network, avoiding the accumulation of damaged contents in mitochondria [12,15]. Interestingly, Kowald and Kirkwood have proposed mitochondrial fusion as being a permissive mechanism to clonal expansion of mitochondrial deletion mutants, rather than a rescue mechanism for damaged mitochondria [16,17].

Dynamin-related protein 1 (DRP1), a member of the dynamin family of guanosine triphosphatases (GTPases), is the key component of the mitochondrial fission machinery [18]. Dynamin-related protein 1 has been linked to the development of different malignant tumors, including skin, brain, breast, lung, thyroid and endometrial cancer. However, the underlying mechanism(s) for this association is still being explored [19–24]. Dynamin-related protein 1 had roles in changing cellular metabolism in melanoma, contributing to stemness in glioblastoma, involvement with lymph node metastases in breast cancer, sustaining cell cycle and proliferation in lung cancer, and associations with the oncocytic phenotype in thyroid cancer [19–23]. Besides its impact on metabolic regulation, DRP1 has also been associated with a broad range of cell processes: apoptosis, mitochondrial biogenesis and mitophagy, cell proliferation, and differentiation and transformation [19,25–29].

Herein, we review the published knowledge on the role of DRP1 in cancer, exploring its interactions with different biological processes, particularly in the tumorigenesis context. Given the broad range of cellular processes where DRP1 is involved, and its interactions with key known hallmarks of cancer, we will start by reviewing DRP1 role in mitochondria fission and its regulation. Following this, we will provide an overview of DRP1 interplay with biological processes known to be altered in cancer which are important for tumor progression, such as cell death, metabolic programming, and the cell cycle (Table 1). We will then discuss dysregulation of these processes in different tumor models centered on DRP1 alterations, particularly the role of this protein in the invasion and metastization processes, relevant for the generalization stages of tumorigenesis. We will finish with a summary of future perspectives and potential clinical implications of targeting DRP1.

2. Regulation of Dynamin-Related Protein 1 and Its Central Role in Mitochondrial Fission

Mitochondrial fusion and fission proteins, first identified in flies and yeast, are key players in mitochondrial biogenesis [30]. There are three highly conserved dynamin-related GTPases (DRPs) regulating membrane dynamics in various cellular processes. These large proteins contain a canonical GTPase domain and various regions that enhance self-assembly via both intra- and inter-molecular interactions [31]. The mitochondrial fission components were first described in yeast genetic screening studies [32]. Dynamin 1 protein (Dnm1) is structurally related to the large dynamin family and was the first protein to have shown a clear role in controlling mitochondrial fission and morphology in *Saccharomyces cerevisiae* [33,34]. In 1998, Otsuga et al. have shown, in yeast, that dynamin-1-like gene (*DNML1*) mutants, with defects in the predicted GTP-binding domain, had a markedly distorted mitochondrial morphology and an altered network distribution, associated with the impairment of mitochondrial fission [33,34]. Around the same time, the human ortholog of dynamin-1-like protein

(DNML1)- DRP1 - was described and was shown to be essential, and the main driver for mitochondrial division in mammalian cells [35,36].

Although DRP1 is described as being mostly a cytoplasmic protein, it has been detected both in cytosol and mitochondria in baseline conditions [19–23]. Indeed, DRP1 translocates to mitochondria upon activation of a stimulus, such as mitochondrial membrane uncoupling, where it links to receptors such as mitochondrial fission factor (MFF) and fission 1 protein (FIS1), constricting the outer mitochondrial membrane in a process dependent on GTPase activity [17]. While MFF is required for DRP1 recruitment, it should be noted that different studies have questioned the role of FIS1 in inducing mitochondrial fission [35–39]. Depending on the cell types and conditions other proteins, such as mitochondrial protein of 18 kDa (MTP18), ganglioside-induced differentiation-associated protein 1 (GDAP1), mitochondrial dynamics protein of 49 kDa and 51 kDa (MiD49 and MiD51), or mitochondrial elongation factor 1 (MIEF1) have a role in cytoplasmically-localized DRP1 activation needed for its recruitment to mitochondria fission sites [38,40,41]. Ganglioside-induced differentiation-associated protein 1 is mainly expressed in neurons and Schwann cells [42]. Additionally, endophilin was reported to act downstream of DRP1 and to be important in the maintenance of mitochondrial morphology [43].

Dynamin-related protein 1 assembles in spirals at sites where endoplasmic reticulum tubules cross over mitochondria and subsequent actin polymerization by inverted formin-2 (INF2) occurs, ultimately leading to mitochondrial fission, as depicted in Figure 1 [44]. Since localization of DRP1 and MFF is dependent on nucleoids, known to be structures composed of both mtDNA and proteins putatively involved in the replication of mtDNA, mitochondrial fission often occurs adjacent to nucleoids [45].

Of note, DRP1 overexpression does not lead to mitochondrial fission *per se*, since DRP1 activity is dependent on its activation by different post-translational modifications, and on the translocation from cytosol to mitochondria. These modifications may include phosphorylation, SUMOylation, ubiquitination, *S*-Nitrosylation and *O*-GlucNAcylation [46–49]. This fact should be kept in mind when interpreting the data described in the literature. The translocation of DRP1 from cytosol to mitochondria may also be impaired by GTPase domain mutations leading to defects in higher-ordered assembly [50]. Several kinases control DRP1 activity by phosphorylation at 3 main sites—Ser616, Ser637 and Ser693 [49,51–56]. The phosphorylation of $DRP1^{S616}$ can be made by different protein kinases involved in signaling pathways, cell cycle, cell cytoskeleton, or Ca^{2+} signaling. These include protein kinase C (PKC), CDK1/Cyclin B in the context of mitosis, rho-associated coiled-coil kinase (ROCK) or Ca^{2+}/calmodulin-dependent protein kinase (CAMK -Iα), to promote fission [51,54,57]. On the other hand, phosphorylation of $DRP1^{S637}$, namely by protein kinase A (PKA), inhibits fission [51–53]. Opposite to this, dephosphorylation of $DRP1^{S637}$ by calcineurin, which is activated by mitochondrial depolarization and by sustained cytosolic calcium increase, including in situations of starvation and apoptosis stimuli, promotes mitochondrial fission [57]. Finally, phosphorylation of $DRP1^{S693}$ by glycogen synthase kinase 3β (GSK3β), a negative regulator of glycogenesis and a known regulator of various signaling pathways and cellular functions, has been demonstrated to prevent fission during apoptosis [49]. Several cancer signaling pathways involving PKA, AMP-activated protein kinase (AMPK) and epidermal growth factor receptor-retrovirus associated sequence oncogene signaling pathway (EGFR-RAS) activate DRP1 driven mitochondrial fission, as will be discussed later [19,28,29,58–61]. On the other hand, after induction of macroautophagy by starvation, mitochondria elongate both in vitro and in vivo [62]. Starvation induces an increase in cyclic adenosine monophosphate (cAMP) levels and leads to PKA activation which contributes to a more effective ATP production through mitochondria elongation [63]. For a more in-depth review of the fission and fusion machinery please refer to Silva et al. [17].

Table 1. Summary of Dynamin-related protein 1 (DRP1) interplay with key cellular processes.

Cell Process	Effects
Cell Death	• DRP1 associates with bcl-2-associated X protein (BAX) at mitochondrial fission sites, promoting permeabilization of the outer mitochondrial membrane (OMM) and cytochrome *c* release [64] • DRP1 drives balance between fission-fusion impacting mitochondrial Ca^{2+} responses in apoptotic signaling [65] • DRP1 inhibition inhibits BAX-BAK dependent cytochrome *c* release [66] • DRP1 knockdown reduces caspase-3 activation and apoptosis [67] • DRP1 inhibition is associated with increase in apoptosis [22]
Metabolic Reprogramming	• DRP1 upregulation associates with less metabolically active mitochondria and increased mitochondrial biogenesis [68] • DRP1 inhibition associates with increased mitochondria oxidative capacity [68]
	Response to hypoxic conditions: • DRP1 expression increased [69] • DRP1 expression decreased after inhibition of HIF-1α [69] • DRP1 inhibition affects HIF1-α expression [69]
	Response to starvation: • Decrease in mitochondrial fraction and activation of $DRP1^{S616}$ through PKA activation [53,70] • Elongation of mitochondria [70] • Shift from glycolysis to oxidative phosphorylation (OXPHOS) [70] • Activation of LDH-A and PDK1 HIF-1α target genes [70] • OXPHOS/glycolysis interchange through HIF-1α /c-MYC pathway [71]
Cell Cycle	• DRP1 functionally or molecularly linked to Cyclin B, E and D [19,29,54,55,72,73] • DRP1 correlates with cell-cycle genes in various cancer types [74] • Mitochondrial morphology is associated with cell cycle control at the G1–S boundary [29,54] • DRP1 inhibition is associated with decrease of cell viability and mitotic program [29,54] • DRP1 knockdown reduces proliferation and percentage of cells in sub-G_0/G_1 cell cycle phase [67] • DRP1 downregulation associates with activation of DNA damage signaling pathways and ATM kinase-dependent G_2/M cell cycle checkpoint, genomic instability and aneuploidy [28] • DRP1 inhibition significantly decreases tumor size [22]

BAX: Bcl-2-associated X protein; BAK: Bcl-2-associated death promoter protein; HIF1-α: hypoxia-inducible factor 1; PKA: protein kinase A; LDH-A: lactate dehydrogenase A; PDK1: pyruvate dehydrogenase kinase 1; c-MYC: myelocytomatosis oncogene protein; ATM: ataxia telangiectasia mutated protein.

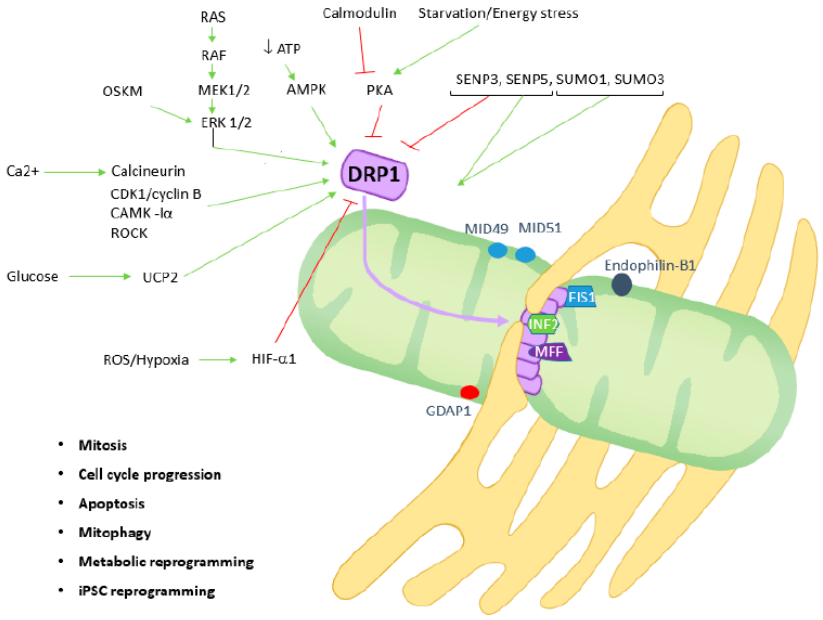

Figure 1. Key players and stimuli in DRP1-mediated mitochondrial fission, both in physiologic and tumor conditions. Green arrows represent stimulation or activation of pathway; red arrows represent repression or inactivation of pathway. SUMO1/Sentrin/SMT3 specific peptidase 3 and 5 (SENP3 and SENP5) and small ubiquitin-like modifier and small ubiquitin-like modifier 1 (SUMO and SUMO1). SENP are deSUMOylating enzymes. For a more in-depth review of the fission and fusion machinery please refer to Silva et al. [17].

3. Dynamin-Related Protein 1 and Cell Death

Mitochondrial division and fusion regulate mitochondrial-dependent intrinsic apoptosis, which relies on the mitochondrial outer membrane permeabilization (MOMP) and in mediators of cell death, such as cytochrome *c*, to be released from the mitochondria [66,75–78]. Mitochondrial fusion protects cells from apoptosis driven by the role of optic atrophy 1 protein (OPA1) in cristae maintenance, which attenuates the MOMP-induced release of cytochrome *c* [79–83]. Mitochondrial fragmentation is known to be involved in several apoptotic models [65]. The role of DRP1 has been detected in complexes with bcl-2-associated X protein (BAX) at mitochondrial fission sites, contributing for the permeabilization of the outer mitochondrial membrane (OMM) and cytochrome *c* release [64]. The role of DRP1 in apoptosis and cell death, as in many other cell biological functions, may seem counterintuitive. Szabadkai et al. have used HeLa cells to overexpress DRP1 and thereby assess the role of mitochondrial division in apoptotic signaling and sub-cellular Ca^{2+} homeostasis [65]. The authors have observed a fragmentation of the mitochondrial network, and a blockage of the intramitochondrial Ca^{2+}-propagating waves [65]. However, the apoptotic effect of ceramide on DRP1 expressing cells was significantly reduced, while sensitivity to staurosporine-induced apoptosis was enhanced, raising the hypothesis that a balance between fusion and fission processes may impact on mitochondrial Ca^{2+} responses [65]. In fact, ceramide acts by inducing Ca^{2+} release from the endoplasmic reticulum (ER) and also to sensitize mitochondria to Ca^{2+} impulse, while staurosporine has a direct effect on the OMM permeabilization [65]. Based on these findings, Szabadkai et al. proposed a model in which DRP1-mediated mitochondrial fission leads to mitochondria positioning far from the ER, thereby reducing the efficiency of Ca^{2+} uptake, which may still be sufficient for normal mitochondrial function, but may serve as a protective mechanism in responses to stress, preventing apoptosis [64]. Other studies have shown that the downregulation or knock-down of DRP1, or the use of mitochondrial division 1 inhibitor (Mdivi-1), widely used as putative specific DRP1 inhibitor, can prevent cell death and/or

promote cell proliferation [22,66,67]. The interpretation of the data published using this compound should take into consideration the caveat of Mdivi-1 not being currently considered as a specific DRP1 inhibitor, but rather as a weak and reversible complex I inhibitor [84]. In particular, Cassidy et al. found that Mdivi-1, retards apoptosis by inhibiting mitochondrial OMM permeabilization and consequently cytochrome *c* release [66]. Rehman et al. have showed that the genetic inhibition, and the use of Mdivi-1, in human lung cancer cell lines led to a decrease in mitochondria fragmentation and a three- to four-fold increase in apoptosis [22]. Finally, Yamauchi-Inoue and Oda have demonstrated that DRP1 knockdown in human colon cancer cells resulted in significantly reduced proliferation, increased percentage of cells in sub-G_0/G_1 cell cycle phase, caspase-3 activation and apoptosis [67]. Interestingly, a reduction in mitochondrial membrane potential was also observed, which may explain the release of cytochrome *c* seen in apoptosis following caspase activation [67].

All this evidence highlights the potential dual role of DRP1 on cell death and cell proliferation. On one hand, DRP1 may act as a gatekeeper, preventing apoptosis under sub-maximum stress conditions; on the other DRP1-driven mitochondrial fission is needed for cell death and cell proliferation to occur, as explained before. These opposing effects will also become obvious in the tumorigenesis section below, where DRP1 expression or activity may reflect pro-apoptotic or pro-proliferative traits, the former being potentially advantageous for therapeutic purposes.

4. Dynamin-Related Protein 1 and Metabolic Reprogramming

The relationship between mitochondrial morphology and cell energetics and survival has already been documented. Mitochondrial elongation increases mitochondrial function and protects cells from apoptosis [62,85]. Cells tend to present mitochondria in an elongated form under starvation conditions, and in a fragmented state under a nutrient-rich environment [62,85]. Mitochondrial elongation contributes to mitochondrial function and protects cells from apoptosis under conditions of starvation in mouse embryonic fibroblasts (MEF) cells [62,86]. Mitochondrial metabolic reprogramming is a hallmark of tumorigenesis, and it has been well described that in most of the tumor cell types, an increase in aerobic glycolysis takes place, a phenomenon known as the Warburg effect [87]. However, it is also recognized that cancer cells can adapt their metabolic profile to their needs. A study that shed light on how mitochondrial morphology links with metabolism plasticity in cancer cells was published by Li et al., who have investigated the changes in mitochondrial morphology induced by nutrition deprivation in tumor cells, using different tumor type cell lines [70]. A dramatic mitochondrial elongation was induced by starvation. This finding was concomitantly associated with a significant decrease in the DRP1 mitochondrial fraction and a dramatic increase in the phosphorylated form $DRP1^{S637}$ driven by PKA activation, proven to be required for the energy stress-induced mitochondrial elongation in hepatocellular cell carcinoma (HCC) cell lines [70]. More importantly, mitochondrial elongation was found to induce a metabolic shift from glycolysis to oxidative phosphorylation during energy stress [70]. Mitochondrial elongation induced by energy stress facilitated cristae formation and the assembly of respiratory chain complexes I–IV to promote oxidative phosphorylation [70]. This, in its turn, led to a negative feedback effect on glycolysis through nicotinamide adenine dinucleotide (NAD^+)-dependent sirtuin 1 (SIRT1) activation, a nutrient-sensing deacetylase [70]. Starvation treatment inhibited the acetylation of hypoxia-inducible factor 1α (HIF-1α) and the expression of pyruvate dehydrogenase kinase 1 (PDK1) and lactate dehydrogenase A (LDH-A), which are known to be HIF-1α target genes. This was reversed by the expression of the mutant $DRP1^{S637A}$, which was associated with mitochondrial fission [70]. This study also indicated that $DRP1^{S637}$-mediated mitochondrial elongation also predicted a poor prognosis in hepatocellular carcinoma patients [70]. Expression of phosphorylated $DRP1^{S637}$ was found to be significantly correlated with larger tumor size, high tumor-node metastasis stage, and a significantly reduced overall survival and recurrence free survival [70]. Consistent with these results, nutrient deprivation was associated with OXPHOS/glycolysis interchange in a human glioma cell line, via HIF-1α/cellular myelocytomatosis oncogene protein (c-MYC) pathway, although a correlation with potential changes in

mitochondrial shape has not been assessed in this study [71]. Interestingly, metabolic reprogramming is also a finding that seems to be associated with precancerous lesions of the colon, where a significant increase in gene expression of *DNML1* was shown, which was accompanied by indirect markers of the Warburg effect in human samples, as reported by Cruz MD et al. [88]. Zou et al. have elucidated how DRP1 dysregulation may interact with mitochondrial biogenesis and mitochondrial autophagy (mitophagy), and thereby with metabolic reprogramming. The authors have assessed the autophagic flux by evaluating the impact of autolysosome inhibitors on the microtubule-associated protein-1 light chain 3α phosphatidylethanolamine conjugate (LC3-II) levels, a protein known to be important for autophagosome formation [68]. They have shown a pattern of DRP1 upregulation, which was associated with metabolically less active mitochondria in a breast cancer cell line. This was accompanied by a reduction in the number of mitochondria, an increase of mitochondrial biogenesis markers such as peroxisome proliferator-activated receptor γ coactivator 1-α (PGC1α), nuclear respiratory factor 1 (NRF1), and mammalian mitochondrial transcription factor (TFAM), and a significant upregulation of B-cell lymphoma 2 protein (BCL-2) nineteen-kilodalton interacting protein 3 (BNIP3), a mitophagy marker, and of the autophagic flux, suggesting an increased mitophagy that explained the reduced number of mitochondria [68]. This pattern was also confirmed in vivo in human breast carcinoma tissue, based on the analyses of a series of human breast cancer from The Cancer Genome Atlas (TCGA database) [68]. Breast cancer cell lines exposed to Mdivi-1 exhibited a reduced autophagic flux and a shift from a glycolytic to an oxidative phenotype, suggesting a reversal of the Warburg effect [68]. The authors suggested a role of DRP1 in the coordinated increase of mitochondrial biogenesis and mitophagy, and in the regulation of breast cancer cell metabolism and survival since a significant decrease of cancer cell viability was also shown. It would be interesting to assess whether these Mdivi-1-induced metabolic effects can be explained by DRP1 inhibition, or through its currently proposed mechanism of action as a reversible Complex I inhibitor [84].

Beyond the effects of starvation in the metabolism of cancer cells, it is also of the utmost relevance to explore the role of hypoxia on metabolic tumor cell adaptation. Using mtDNA-enriched (SK-N-AS) and depleted (ϱ0) cells of neuroblastoma cultured in hypoxic conditions, Kuo et al. have shown that hypoxia-stimulated HIF-1α expression, which was also influenced by the level of reactive oxygen species (ROS), was accompanied by increases of LDH-A and PDK1 as well as an increased expression of DRP1 [69]. Additionally, in mtDNA-enriched cells, a higher expression of DRP1 during hypoxia was observed, which was reverted after genetic suppression of HIF-1α [69]. Indeed, mtDNA seemed to be a mediator of HIF-1α, linking metabolic reprogramming to mitochondrial biogenesis [69].

All these data underscore the role of DRP1 as an indirect mediator of a metabolic shift under starvation conditions, when cancer cells need to rely on a more efficient energy production process (OXPHOS) as opposed to the classic glycolytic phenotype. On the other hand, DRP1 should also be seen as a key linking piece that connects different features of the same process (metabolic reprogramming, to meet cell energy needs, mitochondrial biogenesis, building the cell powerhouse that delivers that energy, and mitophagy, a system that promotes the quality control of mitochondria, as will be seen later). Therefore, depending on the different stimuli and needs, and even depending on specific driver oncogenes, the role of DRP1 is possibly two-pronged: being permissive to OXPHOS or promoting glycolysis.

5. Dynamin-Related Protein 1 and the Cell Cycle

Mitochondrial fission occurs during cellular division, thus securing a proper mitochondrial number in daughter cells. Dynamin-related protein 1 has been described to be functionally or molecularly linked to Cyclin B, E and D [19,29,54,55,72,73]. As previously mentioned, mitochondrial fission during mitosis depends on translocation of DRP1 to mitochondria and phosphorylation of $DRP1^{S616}$ by Cyclin B-CDK1 [89]. On the other hand, mitochondrial shape was found to regulate the cell cycle, as demonstrated by the relationship between the mitochondrial hyperfusion at G_1-S and the Cyclin E buildup needed to entry into S phase [89]. Additionally, DRP1 has been identified as one of the

Cyclin D1-interacting proteins in human tumors, including breast and colorectal cancer [89]. Previous studies have demonstrated that DRP1-driven mitochondrial fission is critical for regulation of cell proliferation in a *Drosophila* model system, as well as in mammalian cells [89]. Mitochondrial function can impact cell cycle regulation; however, this has been an underexplored area in cancer research.

Taguchi et al. have studied mitochondrial dynamics and inheritance in mammalian cells undergoing mitosis in vitro and they showed that mitochondria have a tubular network appearance in interphase cells, moving into fragmented status in early mitotic stage, and going back to filamentous structures in the late phase of mitosis, the mitochondrial fission being a result of $DRP1^{S585}$ phosphorylation by CDK1/Cyclin B [54]. Although the exact mechanism by which fission occurs is not yet totally known, endophilin and probably other downstream factors may play a role [90]. The elongated shape of mitochondria in G_1/S interface is linked to the cellular requirement of ATP and high Cyclin E levels [29,72]. It is therefore thought that throughout the cell cycle, mitochondrial dynamics provides the energy requirements that are needed.

Parone et al. showed that downregulation of DRP1 in HeLa cell lines causes mitochondrial dysfunction, with an increase in ROS levels, a loss of mtDNA, a reduction in cellular ATP, proliferation arrest, and autophagy [91]. It seems therefore that cellular homeostasis is dependent on DRP1-dependent mitochondrial fission. On the other hand, mitochondrial hyperfusion induced by DRP1 deficiency was found to trigger a signaling of replicative stress by which ataxia-telangiectasia mutated/checkpoint kinases 2 and 1 (ATM/Chk2 and ATR/Chk1) DNA damage signaling pathways, as well as the ATM kinase-dependent G_2/M cell cycle checkpoint, are activated [72]. A pattern of genomic instability and aneuploidy in p53 wild type and mutated cells, independent of ATP production defects or ROS production, was also found, suggesting that DRP1 may be implicated in mitochondria-nucleus retrograde signaling and raising the hypothesis that mitochondria play a role in tumorigenesis [72].

Rehman et al. have compared the level of mitochondria fragmentation in several human lung cancer cell lines and normal human cell lines. They observed that all malignant cells presented a markedly higher level of mitochondria fragmentation, which was linked to higher DRP1 and lower mitofusin-2 (MFN2) expression levels, the latter being a protein involved in mitochondrial fusion [22]. The same was observed in lung adenocarcinoma samples, when compared to adjacent normal lung tissue. Additionally, the levels of phosphorylated $DRP1^{S616}$ were also significantly higher, as opposed to phosphorylated $DRP1^{S637}$ which was lower in both lines. The genetic inhibition of DRP1, and the use of Mdivi-1, has led to a decrease in mitochondrial membrane potential, and a decrease in the number of cells in S phase and an increase in number of cells in G_2 phase, again indicating an inhibition of the mitotic program [22]. Both these interventions were also tested in a lung adenocarcinoma xenograft model, leading to a significant decrease in tumor size [22].

Mitra et al. have reported a relationship between mitochondrial morphology and cell cycle control at the G_1–S boundary [29]. Mitochondria change from fragmented structures into a hyperfused state at G_1–S transition. In this stage of the cell cycle, the mitochondrial network presents a greater ATP output than isolated mitochondria at any other cell cycle stage. Hyperfused mitochondria might also play a role in tumorigenesis, since it is known that many cancer cells present dysregulated Cyclin E levels, the cyclin responsible for G_1-to-S phase progression and lose control of G_1–S transition [92,93].

Zhan et al. have shown that the expression of DRP1 increased mitochondrial fission and promoted the proliferation of HCC cells both in vitro and in vivo, by enhancing the G_1/S phase transition [94]. Additionally, the authors have verified that DRP1 knockdown induced a significant G_1 phase arrest in vitro, and reduced tumor growth in vivo [94]. More importantly, they have demonstrated that the promotion of proliferation by DRP1-mediated mitochondrial fission was mediated through p53/p21 and nuclear factor kappa B (NF-κB) /cyclins pathways [94].

Finally, Tanwar et al. have recently published an exploratory analysis of gene expression data from the 31 cancer types in TCGA, showing that DRP1 is predominantly co-expressed with genes involved in the cell cycle, and in gene expression and metabolism, across the majority of the cancer

types [74]. In particular, their investigation on epithelial ovarian cancer (EOC) revealed that DRP1 co-expresses with the cell-cycle module responsible for mitotic transition, which included over 70 genes involved in various phases of cell cycle (G_1 phase, G_1/S transition, S phase, G_2/M transition and M), such as the mitotic transcription factor forkhead box M1 (FoxM1) and its key downstream targets regulating mitotic transition. Inactivation of DRP1 through DRP1 knock-down in EOC cells led to attenuation in mitotic transition [74]. Interestingly, DRP1-cell-cycle co-expression module was detected in epithelial ovarian tumors which responded to chemotherapy, suggesting that DRP1 driven mitosis may contribute to chemo-sensitivity of the primary tumors.

In summary, the pattern of higher DRP1 expression observed in different malignant tumors, as we will later see, seem to indicate a higher proliferative profile of those cells. Complementary to this, DRP1 could also represent a caretaker mechanism, in the sense that its downregulation can trigger the activation of DNA damage signaling pathways, and in an extreme context, ultimately lead to tumorigenesis. The fact that DRP1 is directly involved in cell cycle progression makes it an attractive target for directing therapy agents that interfere with cell proliferation.

6. Dynamin-Related Protein 1 Expression and its Role in Tumorigenesis

DRP1 expression patterns and its role in cancer have been documented in several tumor models and are summarized in Table 2. Wieder et al. described an expression of phosphorylated DRP1^{S616} in nearly half of the cases of a melanoma series, 95.6% of which were *BRAF*^{V600E} tumors [19]. Interestingly, the same relationship with B-Raf proto-oncogene (*BRAF*) status was observed in dysplastic nevi, with 92% of *BRAF*^{V600E} samples being positive for phosphorylated DRP1^{S616} [95]. Genetic inhibition of DRP1 in *BRAF*^{V600E} melanoma cell line led to a loss of expression of DRP1 that was correlated with decreased cell proliferation. On the other hand, the use of Mdivi-1 led to a decrease in DRP1-dependent mitochondrial fission and dose-dependent apoptosis, which was not seen in the wild type (WT) *BRAF*^{WT} melanoma cell line, suggesting that the induction of phosphorylated DRP1^{S616} in dysplastic nevi and in primary melanoma may be a contributing factor to *BRAF*^{V600E} disease, raising the question of its potential role as a prognosis biomarker in this context [95]. These results should take into consideration the caveat of Mdivi-1 not being currently considered a specific DRP1 inhibitor [84,95].

Rehman et al. documented an increase in DRP1 expression in tissue samples from patients with lung adenocarcinoma [22]. An identical pattern was observed in cultured lung cancer cell lines, with increased levels of phosphorylated DRP1^{S616} and decreased levels of phosphorylated DRP1^{S637} [22]. Interestingly, Mdivi-1 was tested in a lung adenocarcinoma xenograft model and proved to significantly reduce tumor size, with an increase in the uptake of 18F-fluorodeoxyglucose (^{18}FDG) in the residual tumor, suggesting an effect on tumor metabolism [22]. Considering the currently proposed mechanism of action of Mdivi-1, as an inhibitor of complex-I and ROS production, it would be interesting to assess if the described reduction of tumor size may be related with potential changes in mitochondrial metabolism.

Table 2. Summary of DRP1 expression patterns and tumorigenic effects in different tumor models.

Tumor Model	DRP1 Expression Pattern and Tumorigenic Effects
Melanoma	• Expression of phosphorylated DRP1^{S616} associated with *BRAF*^{V600E} pre-neoplastic lesions and malignant tumors [95]
Lung Cancer	• Overexpression of DRP1 ex vivo [22] • Increased expression of phosphorylated DRP1^{S616} and decreased levels of phosphorylated DRP1^{S637} in vitro [22]
Breast Cancer	• DRP1 expression associated with invasive tumors and lymph node metastases ex vivo [21,96] • Expression of phosphorylated DRP1^{S616} in vitro [21,96] • Invasion and migration capacities in vitro, including hypoxia-induced [21,96]

Table 2. *Cont.*

Tumor Model	DRP1 Expression Pattern and Tumorigenic Effects
Thyroid Cancer	• Overexpression of DRP1 in oncocytic tumors and oncocytic carcinomas ex vivo [23] • Invasion and migration in vitro [23]
Type I Endometrial Cancer	• DRP1 expression ex vivo associated with mitochondrial dysfunction, anti-apoptotic and anti-oxidant profile [24]
Epithelial Ovarian Cancer	• DRP1 expression ex vivo associated with anti-apoptotic profile [74] • DRP1 driven mitosis linked to chemo-sensitivity of primary tumors [74]
Neuroblastoma	• DRP1 expression and mitochondrial translocation in vitro associated with Survivin anti-apoptotic effects and glycolytic phenotype [97]
Glioblastoma	• Upregulation of DRP1 and hypoxia-induced cell migration in vitro [98]

Ferreira-da-Silva et al. studied benign and malign thyroid tumors, including oncocytomas, which are characterized by a large accumulation of mitochondria in the cytoplasm of their cells [23,99,100]. Interestingly, they found a statistically significant overexpression of DRP1 protein in the oncocytic versus the non-oncocytic thyroid tumors. This pattern was also found when they compared oncocytic carcinomas with oncocytic adenomas [23]. However, the same trend was not observed when comparing benign and malignant tumors overall, nor within the non-oncocytic group of adenomas versus carcinomas. Following these same findings, Ferreira-da-Silva et al. have documented a statistically significant higher expression of DRP1 in an oncocytic thyroid carcinoma cell line compared with a non-oncocytic cell line, an observation that was not explained by differences in mRNA expression [23]. The higher expression of DRP1 was also associated with a more fragmented mitochondrial network [23]. The genetic inhibition of DRP1 reduced cell motility in the oncocytic cell line by close to 50%, a pattern that was also seen with the use of Mdivi-1 [23]. The higher DRP1 expression and fission profile may explain the oncocytic pattern of this particular subset of thyroid tumors, given the known role of DRP1 in mitochondrial biogenesis [23,101]. The association between DRP1 and the potential for higher migration and invasion capacities of the malignant oncocytic tumors is a trait that may also be explained by DRP1 overexpression, and one that has been shown in other tumor models, as later described [23].

Serasinghe et al. have shown that E1A plus RAS^{G12V}-infected MEFs induce DRP1 mRNA expression, DRP1 expression, its activation through phosphorylation of serine 952 residue (murine equivalent of $DRP1^{S616}$ phosphorylation), and a glycolytic phenotype [19]. Through *DRP1* genetic inhibition, and also after the use of Mdivi-1, DRP1 expression and function were found to be required for MAPK/ERK kinase (MEK) triggered transformation when RAS^{G12V} signaling is induced [19]. When they tested two small MEK inhibitors in those transformed cells this led to increased mitochondrial fusion, which was shown to be directly related to the phosphorylation of $DRP1^{S592}$ [19]. Similar results were observed in a human $BRAF^{V600E}$ melanoma cell line, where different upstream and downstream mitogen-activated protein kinase (MAPK) inhibitors have led to mitochondrial fusion, which seemed to be dependent on direct effects in the MAPK pathway, since drug-resistant cell lines were not sensitive to this effect [19]. This result seemed to be independent of mitochondrial biogenesis and was reversible [19]. Similarly, MAPK inhibitors inhibited DRP1 mRNA, protein, and $DRP1^{S616}$ phosphorylation, and led to reprogramming of mitochondrial metabolic function, shifting it to an OXPHOS patterned metabolism [19]. These authors also documented a significantly higher phosphorylated $DRP1^{S616}$ expression rate in $BRAF^{V600E}$ melanoma patient samples when compared with $BRAF^{WT}$ samples [19]. According to Serasinghe et al. experiments, DRP1 seems to regulate mitochondrial function before an oncogenic signaling is initiated, during carcinogenesis and after oncogenic MAPK signaling inhibition [19].

Lennon et al. have specifically explored mitochondrial morphology through fractal dimension and lacunarity measurements in mesothelioma cell lines, as a prediction of responses to treatments

that interfere with mitochondrial metabolism [102]. Fractal dimension and lacunarity are quantitative measurements which allow the description of complex structures, such as mitochondria. The former relies on a mathematical principle of self-similarity between different biological structures, while the latter is based on the texture of a shape. An altered ratio of DRP1-MFN2 in both total cell lysates and mitochondrial fraction was detected, suggesting a higher relative rate of fission as compared to fusion [102]. Interestingly, mitochondrial morphology showed a better correlation with mitochondrial inhibitors sensitivity than did metabolic function [102]. As pointed out by the authors, increased fission seemed to be associated with decreased mitochondrial activity and mitochondrial membrane potential, which could explain an increase in cell death with mitochondrial inhibitors [102].

Hagenbuchner et al. have studied the mitochondrial effects of Survivin, a known anti-apoptotic protein that is overexpressed in neuroblastoma with gain of chromosome 17q, typically associated with high stage cancer, poor prognosis, and chemotherapy resistance [97]. In Survivin-expressing cells, mitochondria presented as punctuated, perinuclear structures, due to an increase in the expression of DRP1, which was accompanied by a reduction in the expression of BCL-2-like protein 11 (BIM) [97]. In these cells, DRP1 localized in mitochondria, but no cytochrome *c* release was observed due to the absence of BIM [97]. These effects were affected through genetic inhibition of DRP1, and also after the used of Mdivi-1 [97]. Curiously, an effect of Survivin on oxidative phosphorylation, through an impact on complex I and IV, was also shown to result from DRP1-induced mitochondrial fission, with no changes in ATP levels, raising the hypothesis that ATP in these Survivin expressing cells may be produced as a result of glycolysis, which was supported by the increase in glucose consumption and lactate production, and by the effect that glycolysis inhibitors had on cell viability reduction and sensitivity to chemotherapy agents [97].

Recently, Guerra et al. have documented an increase in the expression of DRP1 and BNIP3, a molecular mediator which promotes mitophagy, the antioxidant augmenter of liver regeneration (ALR), and the anti-apoptotic molecule BCL-2 in cancer cells of type I endometrial carcinoma with previously described alterations in respiratory complex I (oncocytic-like phenotype), as compared to matched non-malignant tissue and hyperplastic tissue, linking mitochondrial dysfunction to the expression of pro-fission, anti-oxidant, and anti-apoptotic proteins [24].

Tanwar et al. conducted experiments of downregulation of DRP1 in a human ovarian carcinoma cell line, showing a potential causal role of DRP1 in mitotic transition and cell proliferation in EOC cells [74]. These authors have also compared the expression of aldehyde dehydrogenase 1A1 (Aldh1A1), a marker for ovarian cancer stem cells, between primary and relapse tumor samples and have found an inverse relationship between Aldh1A1 and DRP1 expression [74]. This finding suggests that the modulation of DRP1 may potentially be involved in the stem cell properties of the relapsed EOC disease [74]. Based on their results, DRP1 seems to associate with cell cycle acceleration in some relapsed resistant patients (DRP1-High) as compared to others (DRP1-Low) where this does not seem to happen. The authors thereby hypothesize that DRP1 may have a pro-apoptotic role in DRP1-Low and an anti-apoptotic role in DRP1-High patients [74]. Additionally, they have suggested that a DRP1-based-gene expression-signature from primary tumors could stratify patients for survival after exposure to chemotherapy, since the pattern of genes expression seems to differ in both DRP1-High and DRP-Low groups [74].

The RAS-activated molecule recombinant protein of human ralA binding protein 1 (RALBP1) regulates the effect of Cyclin B1 on DRP1 [54,55]. Although RAS-ERK signaling-driven regulation of DRP1 contributes to cell transformation, as previously mentioned, no relationship with cell cycle alteration was found [19,59]. Various studies have implicated extracellular signal-regulated kinase 1 and 2 (ERK1/2) in regulating DRP1 function (Figure 1). Yu et al. have shown that ERK1 could phosphorylate DRP1 in vitro [103]. Gan et al. studied the oxidative stress responses in cytoplasmic hybrid (cybrid) derivatives of neuronal cells, incorporating platelet mitochondria from AD [104]. They showed that ERK1/2 activation driven by oxidative stress increased DRP1 expression and its recruitment to mitochondria, generating increased fission in AD cybrids [104]. However, no functional

link between ERK and DRP1 was established [104]. As mentioned previously, Serasinghe et al. have demonstrated that DRP1^{S616} is phosphorylated by ERK1/2 in cancer cells, promoting mitochondrial fission to support RAS-dependent transformation and tumor growth [19]. When this phosphorylation was reverted in vitro, cells have undergone apoptosis [19]. Recently, Kashatus et al. showed that the expression of mutant *RAS* in HEK cells promoted DRP1-dependent mitochondrial fragmentation [61]. Additionally, knockdown of *DNM1* inhibited the growth of transformed cell tumor xenografts [61]. ERK2 and activated RAS, RAF or MEK mutants were shown to phosphorylate human DRP1^{S616} in vitro, an effect that was abolished by MEK inhibitors [61]. This was accompanied by a reversal of the mitochondrial fission [61].

ERK1/2-dependent DRP1 phosphorylation and mitochondrial fission have been described to induce pluripotent stem cells (iPSCs) during the reprogramming of somatic cells [105]. Prieto et al. have shown that cellular reprogramming into iPSC induced mitochondrial fission early in this process, which was dependent on DRP1 and accompanied by an increase in DRP1 phosphorylation at the murine equivalent of human DRP1^{S616}, with kinetics matching DRP1 recruitment to mitochondria [106]. It was also shown that mitochondrial fission was inhibited by a MEK inhibitor, a pattern which was reverted by a DRP1 phosphomimetic mutant. This raised the hypothesis that ERK signaling may be required for mitochondrial fission early in the reprogramming process [106].

Morita M. et al. have shown that the nutrient-sensing mechanistic/mammalian target of rapamycin complex 1 (mTORC1), which is known to be activated in many different malignant tumors, stimulates the translation of mitochondrial fission process 1 (MTFP1) protein [107]. MTFP1 is, in its turn, associated with phosphorylation and mitochondrial recruitment of DRP1 and a mitochondrial fission pattern [107]. Interestingly, they have found that the suppression of mTORC1 activity led to increased mitochondrial fusion due to the reduced translation of MTFP1, which is mediated by translation initiation factor 4E (eIF4E)- binding proteins (4E-BPs) [107]. The authors further concluded that uncoupling MTFP1 levels from the TORC1/4E-BP pathway after mTOR inhibition blocks the hyperfusion status and leads to apoptosis, thereby offering a new therapeutic opportunity for these type of anti-cancer drugs, converting them from cytostatic to cytotoxic [107].

The mitochondrial uncoupling protein 2 (UCP2) also seems to control mitochondrial fission through DRP1 expression regulation [108,109]. Toda et al. reported mitochondrial changes, such as increase in mitochondrial density and reduction in mitochondrial size, in ventromedial nucleus of the hypothalamus (VMH) neurons mediated by UCP2, suggesting that UCP2 is involved in the regulation of the mitochondrial fission process [110]. In this way, Toda et al. assessed the effect of UCP2 in DRP1 in response to a glucose load and verified a significant increased ratio of phosphorylated DRP1/DRP1 in UCP2 knockout mice with selective re-expression of UCP2 [110]. Interestingly, a few years ago, UCP2 was found to be overexpressed in Hürthle cell tumors [111]. These findings may partially explain the pattern of DRP1 overexpression observed by Silva et al. in Hürthle cell tumors of the thyroid, known to be characterized by at least 75% of oxyphilic cells [23].

7. Role of Mitochondrial Dynamics in Invasion and Metastization

In a series of human breast cancer samples, Zhao et al. observed a significantly increased expression of DRP1 protein in in situ ductal carcinoma in comparison with normal tissue, and in invasive breast cancer and lymph node metastases in comparison with in situ ductal carcinoma [21]. The authors also reported an increased expression of DRP1 and phosphorylated DRP1^{S616} in metastatic breast cancer cell lines, as compared to non-metastatic breast cancer cell lines [21]. DRP1 genetic inhibition led to reduced migration and invasion capacities, which was also verified for cell migration when pharmacological inhibition with Mdivi-1 was tested [21]. Cell cycle or cell viability did not seem to be affected by DRP1 changes [21]. Interestingly, DRP1 silencing led to reduced cell spreading and lamellipodia formation, typically seen in the edge of migrating cells, which was accompanied by a change in mitochondria distribution within the cell, from perinuclear to a more scattered state, independent of the membrane potential [21]. The aforementioned findings suggested that

the upregulation of DRP1 may be an early event in invasive breast cancer development, and formation of lamellipodia is dependent of mitochondria fission [21].

It was demonstrated in a glioblastoma in vitro model that hypoxia induces upregulation of DRP1, mitochondrial fission and cell migration [112–115]. Following these observations, Han et al. looked at the effect of hypoxia in breast cancer cell migration driven by mitochondrial dynamics [20]. Besides the similar pattern of DRP1 expression in metastatic breast cancer cell lines documented before, Han et al. showed that hypoxia led to mitochondrial fission and to a significantly increase in migration of the metastatic cell line in comparison with the non-metastatic cell line. The genetic inhibition of DRP1, as well as the used of Mdivi-1, led to a significant reduction in mitochondrial fission as well as in hypoxia-induced migration [20]. At variance with the non-metastatic cell line, treatment with cisplatin (CDDP) induced apoptosis, mitochondrial fission, increase in intracellular levels of ROS and a decrease in metalloproteinase (MMP) in the metastatic cell line, which was reverted by the inhibition of DRP1 [96]. These results indicate that mitochondrial fission driven by DRP1 induces the metastatic cell line to become more sensitive to cisplatin in hypoxic conditions, potentially but not only through the impact on intracellular ROS and MMP, an effect that was not observed in the non-metastatic cell line [96].

Finally, a study that has shed some light onto the mechanisms that link cell motility and migration with mitochondria and OXPHOS dysfunction, has been published by our group [116]. We have shown that cybrid cells harboring a specific mtDNA mutation are less prone to apoptosis, have a higher motility and migration ability, and produce larger tumors and more lung metastases in a mouse model in comparison with wild-type cells [116].

8. Future Perspectives and Clinical Implications

The role of DRP1 in key hallmarks of cancer, as cell proliferation and survival, apoptosis failure, metabolic reprogramming, invasion and metastization, and even insensitivity to anti-growth or anti-proliferative signals, depends most likely from the interplay between microenvironment stimuli, cells' genetic background, cytotoxic or targeted treatment strategies, and the tumor cell's continuous adaptation to all of these factors. In other words, we may look at DRP1 as a key molecular link between several biological cell processes, which acts as a key player in the plasticity of tumoral cells under various internal and external contexts (Figure 2). This concept has implications both on the interpretation of its biological significance at any given moment of the tumorigenesis process, as well as on the potential effects of its inhibition which can also be paradoxical. As an example, Szabadaki et al. have shown that DRP1 overexpression can prevent apoptosis, but it had a negative effect on cancer survival following MAPK inhibitors [19]. There is evidence suggesting that some tumor cells may become dependent on ERK1/2-driven DRP1 phosphorylation, thus indicating that DRP1 inhibition may be a potential therapeutic strategy for such tumors [104]. Others, however, have demonstrated that DRP1 inhibition can prevent cell death and promote proliferation [29,65,66].

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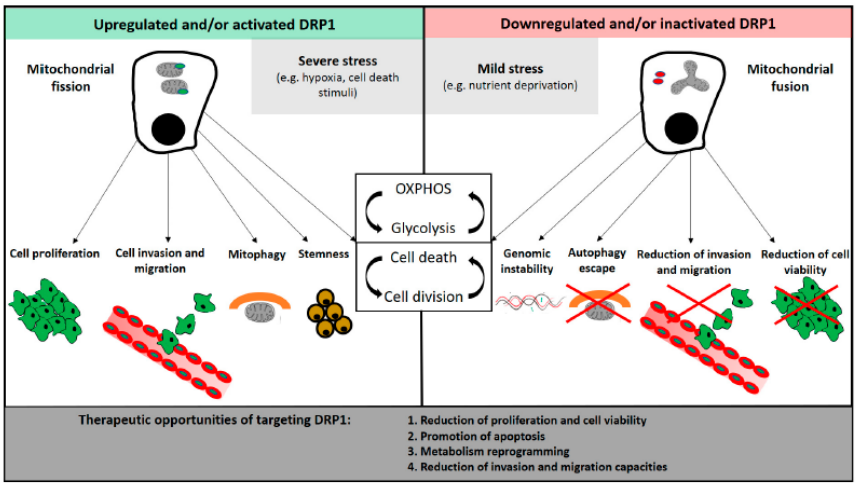

Figure 2. Effects of DRP1 activation and/or upregulation, and associated mitochondrial fission patterns, on tumorigenesis. Inactivation and/or downregulation of DRP1 may have a counteracting effect on tumorigenesis, which could be used as a therapeutic approach in cancer. The effects of both DRP1 activation and inactivation on metabolism reprogramming, and on cell cycle and cell death, should be seen as a continuously dynamic adaptive mechanism to internal and external challenges.

Some of the research presented in this revision suggest a new concept, in which mitochondrial-targeted cancer therapy could be additive to or synergized with therapies that address cancer cell proliferation, such as promoting mitochondrial glucose oxidation [19].

It remains important that the link between DRP1 and cell cycle is better understood. Mitra et al. have found that the G_1–S transition and Cyclin E levels can be regulated by the mitochondrial state, thereby opening new areas of exploration relating mitochondria with cancer [29]. Zou et al. have stressed the emerging evidence of PGC1α contributing to tumor growth, and therefore have proposed the critical importance to target both mitochondrial biogenesis and mitophagy for effective cancer treatment, a concept to be tested in future research as a means to test effectiveness for breast cancer treatment [68]. Additionally, the definition of a relationship between HIF-1α and DRP1 may be of relevance to assess its clinical applications in the future [71].

Finally, we believe it is worthwhile to stress the research recently published by Tanwar et al. [74]. Their DRP1-based analysis highlights that DRP1-driven cell cycle regulation is present in several cancer types, which may allow response to therapies targeting proliferating cells [74]. In particular, their results point out to an important role of mitochondria in ovarian cancer chemo-resistance and relapse [74].

To address the issue on how DRP1 can be targeted, it is important to highlight that, although Mdivi-1 has been widely used as a putative DRP1 inhibitor in vitro and in vivo, including in much of the published data referenced in this review, a recent report has proposed an alternative mechanism of action for this compound, as a reversible mitochondrial complex I inhibitor, not impairing Drp1 GTPase activity. Targeting DRP1 in the context of cancer still seems a promising approach, but not without the challenges of designing and developing compounds that specifically inhibit GTPase activity, and of the complex interplay between mitochondria dynamics and cell requirements in every stage of tumorigenesis.

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ORIGINAL ARTICLE

S616-p-DRP1 associates with locally invasive behavior of follicular cell-derived thyroid carcinoma

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Abstract

Purpose Dynamin-related protein 1 (DRP1), a mitochondrial fission protein, and its active form phosphorylated at Serine 616 (S616-p-DRP1) have been increasingly associated with tumorigenesis and invasion in various tumor models, including oncocytic thyroid cancer (TC). In this study, the expression of DRP1 and S616-p-DRP1 and its relationship with patients' clinicopathological characteristics, tumor genetic profiles, and clinical outcomes were assessed in a large series of follicular cell-derived TC (FCDTC).

Methods Retrospective biomarker study characterizing the clinicopathological and immunochemistry DRP1 and S616-p-DRP1 expression of a series of 259 patients with FCDTC followed in two University Hospitals.

Results DRP1 expression was positive in 65.3% (169/259) of the cases, while the expression of the S616-p-DRP1 was positive in only 17.3% (17/98). DRP1-positive expression was significantly associated with differentiated tumors (67.7 vs. 48.0%; $P = 0.049$), non-encapsulated tumors (73.8 vs. 57.4%; $P = 0.011$) and thyroid capsule invasion (73.4 vs. 57.5%; $P = 0.013$). S616-p-DRP1-positive expression was significantly associated with tumor infiltrative margins (88.9 vs. 11.1%; $P = 0.033$), thyroid capsule invasion (29.8 vs. 3.1%; $P = 0.043$), lymph node metastases (23.3 vs. 8.1%; $P = 0.012$), and higher mean cumulative radioiodine dosage (317.4 ± 265.0 mCi vs. 202.5 ± 217.7 mCi; $P = 0.038$). S616-p-DRP1 expression was negatively associated with oncocytic phenotype (0.0 vs. 26.2%; $P = 0.028$).

Conclusions S616-p-DRP1 is a better candidate than DRP1 to identify tumors with locally invasive behavior. Prospective studies should be pursued to assess S616-p-DRP1 role as a molecular marker of malignancy in TC and in patients' risk assessment.

Keywords Dynamin-related protein 1 · Mitochondrial dynamics · Thyroid cancer, Hürthle cell · Thyroid neoplasms, Oncocytic · Invasion

Introduction

Thyroid cancer (TC) is the most common endocrine malignancy, with a worldwide 5-year prevalence among all

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cancers of 4.6% by 2018 (https://gco.iarc.fr/today/online-analysis-pie, accessed July 12, 2020), ranking 9th place in the list of more common malignancies [1]. TC presents a 3:1 higher incidence in women and includes diseases with remarkably different features varying from indolent localized papillary carcinoma to lethal anaplastic carcinoma [1, 2]. It has been argued that the prognosis of TC depends more on the interplay between clinical and biological factors, including age, size, gender, histopathological features, and genetic factors, than from genetic factors alone [3–6]. Both the International Union Against Cancer/American Joint Committee on Cancer staging system, which combines age and Tumor, Node, Metastases (TNM) staging to assess the risk of death due to TC, and clinico-pathologic features are accepted as prognostic indicators in TC. Less consensus exists about the role of genetic or molecular markers as individual prognosis measure. Amongst these, mutations in the telomerase reverse transcriptase (*TERT*) promoter and in *TP53* have been retrospectively associated with a worse clinical outcome, but still require a prospective validation [7–9]. B-Raf proto-oncogene (*BRAF*) and rat sarcoma viral oncogene (RAS) may also have a prognosis value under some circumstances, not yet fully clarified [3, 5].

Recently, our group reported an overall increase in the levels of "mitochondria-shaping" proteins in TC, suggesting a role for abnormal mitochondrial biogenesis and dynamics in thyroid cell transformation [10]. From those, dynamin-related protein 1 (DRP1)—the major player in mitochondrial fission—was the most highly expressed in TC [10]. DRP1 is the largest member of the dynamin family of guanosine triphosphatase proteinases known to constrict membranes [11–14]. It is mainly a cytosolic protein, but it translocates to mitochondria to promote mitochondrial fission after undergoing extensive posttranslational modifications altering its localization and affinity for oligomerization [12, 13, 15]. DRP1 oligomerizes into spirals around the mitochondrial outer membrane, constricting the organelle through guanosine triphosphatase protein hydrolysis to promote mitochondrial fragmentation [13, 15, 16]. Mitochondria division is needed in different and sometimes opposing processes, such as apoptosis and cell cycle progression, mitosis, as well as in mitophagy [17, 18]. This is achieved, at least partially, through the phosphorylation of DRP1 at serine residue 616—S6161-p-DRP1 [18, 19].

The increased or enhanced activation of DRP1 has been associated with malignant phenotype in various epithelial and endocrine tumors [20–34]. DRP1-based changes in mitochondrial dynamics have been associated with cell migration and invasion in TC, breast cancer, lung cancer and glioblastoma [10, 21, 23, 26]. In TC, overexpression of DRP1 was also found to be associated with oncocytic tumors and, within these, with carcinoma [10]. Interestingly, a higher expression of S616-p-DRP1 has been reported in $BRAF^{V600E}$ mutated melanoma, and in ERK2-activated pancreatic cancer, with mechanistic work supporting the importance of this phosphorylation in tumorigenesis [29, 33–35]. Mitogen-activated protein kinase (MAPK) has been identified as a key signaling pathway involved in DRP1 activation, with ERK1/ERK2 directly phosphorylating DRP1 [29, 33]. When MAPK was inhibited, S616-p-DRP1, but not total DRP1, was reduced, supporting the translational importance of assessing this active form as opposed to total DRP1 in tumor samples [34]. Although most published studies reported on DRP1 expression, recent research has focused on S616-p-DRP1 assessment [20, 23, 29, 34].

Few studies have examined the significance of DRP1 overexpression in TC cells, and in the oncocytic variants in particular [10]. It would be clinically relevant to assess if DRP1—or its activated form S616-p-DRP1—could have a role as a prognosis factor in the risk assessment of patients with DTC, an unmet medical need in those 5–10% of TC cases which will potentially have a poor outcome.

The aim of the present study was to assess the expression of DRP1 by immunohistochemistry (IHC) in a large series of patients with FCDTC, including oncocytic variants, which according with the new classification includes Hürthle cell carcinomas (HCC) [36], and to evaluate the relationship between its expression and the patients' clinicopathological characteristics, genetic or molecular profiles of the tumor, and clinical outcomes. We have also assessed the expression of S616-p-DRP1 in a sub-sample of our series and derived the same analyses described for DRP1.

Material and methods

Tumor samples

Our study included a series of 259 formalin-fixed, paraffin-embedded human tissue samples from FCDTC collected from the biobank of the Institute of Molecular Pathology and Immunology of the University of Porto. The material used in this study originated from patients diagnosed between 1971 and 2010 and followed in two University Hospitals in Portugal (*Centro Hospitalar São João* and *Centro Hospitalar e Universitário de Coimbra*) for whom clinco-pathological data were available, and from the clinical databases of these hospitals. This work was approved by the Ethic Committee for Health (CES) of the Hospital Center of São João/Faculty of Medicine of the University of Porto (CES 137 284–13) and by the Ethic Committee of the Faculty of Medicine of the University of Coimbra (n° 1309). All the procedures described in this study were done in accordance with national ethical standards (Law n° 12/2005) and Helsinki declaration. Samples were collected

upon written informed consent of the patients, or their guardians, in the case of patients under 18 years. In addition, for all clinicopathological data from patients of *Centro Hospitalar São João*, clinical data reuse permission was requested and a DAtaREuseCertificate for Research was issued with the number RAI 17003858 (http://portal-chsj.min-saude.pt/pages/727). All collected data have been analyzed and stored according to the local legal requirements, thereby ensuring the privacy and protection of individual patient data.

The demographic and clinicopathological data of the patients were retrospectively collected from the histopathological reports and clinical databases. The histology of all tumor samples was reviewed independently by two pathologists (ER and MSS), and the thyroid tumor classification was performed according to the 2004 WHO criteria [2]. Patients were stratified by clinicopathological characteristics in the following categories: gender, age (≥45 or <45 years), histological diagnosis, TNM [37] stage, tumor size, tumor capsule invasion, vascular invasion, thyroid capsule invasion, extrathyroidal invasion, multifocality, presence of lymph node and distant metastases, presence of $BRAF^{V600E}$ mutations, presence of *TERT* promoter mutations (−124G>A and −146G>A), cumulative dosage of radioiodine treatment, persistence of the disease at the end of follow-up, disease-specific mortality, and overall mortality. "Aggressive variants" were defined as all cases of solid/trabecular, diffuse sclerosing, tall cell or columnar cell PTC, as well as all cases of PDTC. When comparing PTC with FTC and FVPTC, "aggressive variants" were excluded. We performed an analysis for the whole sample and a subanalysis for the major histotypes. Considering that FTC and FVPTC share morphological (follicular pattern) and molecular features (high proportion of *RAS* mutations), we also considered a subgroup encompassing these two types of tumors [38]. Tumor growth pattern at the tumor margin was evaluated with low-power magnification and categorized as expansive, intermediate or infiltrative, when tumor margin was pushing and reasonably well-circumscribed, when clusters or cords of tumor cells invaded the normal adjacent tissue without distinct border, or when tumor showed both patterns, respectively.

Immunohistochemical analysis

IHC was performed in 3-µm formalin-fixed, paraffin-embedded sections of the 259 tumor samples for DRP1, and in 98 representative tumor samples which were still available for additional S616-p-DRP1 assays. Sections were deparaffinized and rehydrated in a series of decreasing concentrations of ethanol solutions. Deparaffinized sections were subject to heat-induced antigen retrieval in 1 mM pH 9.0 ethylenediaminetetraacetic acid buffer (LabVision Corporation, Fremont, CA, USA). Endogenous peroxidase activity was blocked with UltraVision Hydrogen Peroxide Block and nonspecific bind was blocked using UltraVision Block reagent from UltraVision Quanto Detection System HRP DAB (Thermo Scientific/Lab Vision, Fremont, USA) for 10 min. The sections were then incubated in a humidified chamber, according to the manufacturer's specifications, with the following primary antibodies: mouse monoclonal antibody for DRP1 (1:100) ref. 611112 (BD Biosciences), rabbit polyclonal antibody for S616-p-DRP1 (1:500) ref. 3455, from Cell Signaling. The sections were then washed and stained by using the UltraVision Quanto Detection System HRP DAB (Thermo Scientific/Lab Vision, Fremont, USA). All sections were counterstained with Mayer's hematoxylin. Positive controls from previously tested kidney samples were used in every run. To assess the specificity of the immunostaining, tumor sections not incubated with the primary antibody were used as negative controls. Whenever present, scattered macrophages and muscular tissue were considered as internal positive controls for both proteins. A second internal and positive control for each tumor sample was the expression of both proteins in normal adjacent thyroid tissue.

Evaluation of immunohistochemical staining

Immunostaining was semi-quantitatively evaluated by three observers (ARL, LS, and VM for DRP1 and SC, CT, and VM for S616-p-DRP1) without the knowledge of any clinical information of the cases. The expression of DRP1 in tumor tissue was evaluated according to an immunereactive staining score (IRS) adapted from other studies [39–41]. Immunohistochemical positivity was defined as cytoplasmic staining for DRP1 and S616-p-DRP1, and immunostaining scores were based on the intensity and the extension of tumor cells immunostaining, as described in Table 1. A total IRS was then obtained by multiplying the intensity (I) and extension (E) scores, i.e., $IRS = I \times E$, ranging from 0 to 12. Positive expression or overexpression of DRP1 and S616-p-DRP1 in TC sections was defined by an IRS of 6 or higher. This positivity criterion was based on previous observations that the immunostaining of DRP1 in normal adjacent thyroid tissue was usually weaker than in neoplastic tissue, with an IRS score of 4 or lower.

Statistical analysis

Continuous variables were summarized by mean and standard deviation and compared using Student's *t*-test or by median and minimum–maximum and compared using Wilcoxon's test. Shapiro–Wilk test was used to assess the normality of continuous variables. Categorical variables were summarized by number of cases and percentage and

Table 1 Scoring system for the immunostaining of DRP1 and S616-p-DRP1 in thyroid cancer sections

Intensity (I)		Extension (E)	
Staining strength	Score	% of stained tumor cells	Score
Absent	0	≤10	0
Weak	1	11–25	1
Moderate	2	26–50	2
Strong	3	51–75	3
		>75	4

DRP1 dynamin-related protein 1, *S616-p-DRP1* serine 616-phosphorylated DRP1

compared using Chi-square test or Fisher's exact test, as applicable. All analyses were conducted considering the complete cases for the variables analyzed. To assess the strength of the relationship between DRP1 and S616-p-DRP1-positive cases and other categorical variables, the odds ratio and respective 95% confidence intervals were calculated. The Kaplan–Meier survival curves were plotted with the log-rank test statistics. The Kendal's correlation coefficient was calculated for the total DRP1 score and S616-p-DRP1 score. All statistical analyses were conducted using R Statistical Software (version 3.6.1) and the statistical significance level was set at 5%.

Results

Patient characteristics and clinicopathological variables

Table 2 summarizes the patient and tumor characteristics. This study included samples from patients aged 11–83 years, 80.3% of whom were females. The TC cases included 162 cases of PTC (8 oncocytic and 154 non-oncocytic), 63 cases of follicular variant of PTC (FVPTC, 10 oncocytic and 53 non-oncocytic), 25 cases of FTC (11 oncocytic and 14 non-oncocytic), and 9 cases of PDTC (2 oncocytic and 7 non-oncocytic) (Table 2 and Fig. 1). Most tumors were classified as stage I (47.1%). From the tumors for which data were available, 50.2% were capsulated, of which 80.0% presented capsule invasion. Vascular invasion was present in 35.9%, 49.1% had thyroid capsule invasion, and 37.0% had extrathyroidal extension. Lymph node involvement was present in 36.2% of the patients, and 13.6% had distant metastases during follow-up. Overall, 40.9% of the tumors assessed for mutations presented $BRAF^{V600E}$ mutation, with 51.4% in PTC cases, and 12.3% presented *TERT* promoter mutation. The majority of patients (84.6%) were treated with radioiodine, with a mean cumulative dose of 195.1 ± 235.1 millicurie (mCi). The median follow-up time for all patients was 7.5 (0.1–38.9) years. At the time of the last follow-up, 28.8% of patients had persistent disease and 6.6% patients had died, 55.9% of which due to TC.

DRP1 expression in FCDTC and correlation with S616-p-DRP1

DRP1 expression was positive in 65.3% (169/259) of TC cases in this study (Table 2). Out of the 98 TC cases tested for S616-p-DRP1, 17.3% (17/98) were positive for the expression of the active form of DRP1. Both DRP1 and S616-p-DRP1 antibodies showed cytoplasmic staining with no nuclear staining (Fig. 2a, b). Critically, unlike DRP1, p-616-DRP1 did not stain normal thyroid tissue.

No significant correlation was found between the expression of total DRP1 and S616-p-DRP1 (Kendall's Tau correlation coefficient = 0.063, $P = 0.451$) (Fig. 2c, d). Despite this, the expression of both DRP1 and S616-p-DRP1 was positively associated with more differentiated histologies and locally invasive traits, as described below.

DRP1 expression according to clinicopathological characteristics

DRP1 was positive in 71.0, 64.0, and 44.4% for PTC, FTC, and PDTC, respectively. No differences in DRP1 expression were found between PTC and FTC. However, classic PTC presented a significantly higher proportion of DRP1-positive cases when compared with FVPTC and FTC combined (73.8 vs. 56.9%; $P = 0.007$) (Fig. 3a). Similarly, the proportion of DRP1-positive cases was significantly higher in "non-aggressive" compared with "aggressive" variants (67.7 vs. 48.0%; $P = 0.049$), which corresponded to 25 cases of PTC (Fig. 3b). No associations were found between DRP1 expression and the presence of oncocytic variants (71.9 vs. 64.7%; $P = 0.422$].

Non-encapsulated tumors had a significantly higher rate of DRP1 positivity in comparison with encapsulated tumors (73.8 vs. 57.4%; $P = 0.011$) (Fig. 3c). Tumors with thyroid capsule invasion had a significantly higher rate of DRP1 positivity (73.4 vs. 57.5%; $P = 0.013$) (Fig. 3d).

Patients subjected to more than one radioiodine treatment harbored tumors with significantly lower DRP1 expression (30.8 vs. 69.2%; $P = 0.022$) (Fig. 4).

No significant association was found between DRP1 expression and gender, age, tumor stage, tumor size, vascular invasion, extrathyroidal invasion, multifocality, lymph node or distant metastases, $BRAF^{V600E}$ mutation, *TERT* promoter mutation, cumulative radioiodine dose, disease status at the end of follow-up, disease-related mortality or overall mortality (Supplementary Table 1 and Supplementary Figs. 1 and 2).

Table 2 Characteristics of patients and tumors

Clinicopathological characteristics	DRP1 IRS score			S616-p-DRP1 IRS score		
	Total sample (n = 259, 100%)	Negative[a] (n = 90, 34.7%)	Positive[b] (n = 169, 65.3%)	Total sample (n = 98)	Negative[a] (n = 81, 82.7%)	Positive[b] (n = 17, 17.3%)
Age at diagnosis, years						
≥45, n (%)	135 (52.1)	46 (34.1)	89 (65.9)	59	53 (89.8)	6 (10.2)
<45, n (%)	124 (47.9)	44 (35.5)	80 (64.5)	39	28 (71.8)	11 (28.2)
Mean ± SD	45.5 ± 16.3	45.9 ± 17.8	45.3 ± 15.5	48.1 ± 16.5	49.8 ± 16.1	39.9 ± 17.4
Gender, n (%)						
Female	208 (80.3)	69 (33.2)	139 (66.8)	73	61 (83.6)	12 (16.4)
Male	51 (19.7)	21 (41.2)	30 (58.8)	25	20 (80)	5 (20)
Histological diagnosis, n (%)						
PTC	162 (62.5)	47 (29)	115 (71.0)	66	58 (87.9)	8 (12.1)
FVPTC	63 (24.3)	29 (46)	34 (54.0)	13	11 (84.6)	2 (15.4)
FTC	25 (9.7)	9 (36)	16 (64.0)	13	13 (100)	0 (0)
PDTC	9 (3.5)	5 (55.6)	4 (44.4)	6	5 (83.3)	1 (16.7)
Oncocytic variants, n (%)[a]						
n	247	85 (34.4)	162 (65.6)	94	77 (81.9)	17 (18.1)
Yes	32 (13.0)	9 (28.1)	23 (71.9)	29	29 (100)	0 (0)
No	215 (87.0)	76 (35.3)	139 (64.7)	65	48 (73.8)	17 (26.2)
TNM stage, n (%)						
I	122 (47.1)	39 (32)	83 (68.0)	39	31 (79.5)	8 (20.5)
II	24 (9.3)	8 (33.3)	16 (66.7)	5	5 (100)	0 (0)
III	54 (20.8)	18 (33.3)	36 (66.7)	23	19 (82.6)	4 (17.4)
IV	59 (22.8)	25 (42.4)	34 (57.6)	31	26 (83.9)	5 (16.1)
Tumor size						
n	251	85 (33.9)	166 (66.1)	94	79 (84)	15 (16)
Mean ± SD, cm	27.2 ± 16.4	28.9 ± 18.1	25.8 ± 15.3	29.9 ± 17.8	31.3 ± 18.3	23.0 ± 7.3
≤4 cm	208 (82.9)	67 (32.2)	141 (67.8)	74	60 (81.1)	14 (18.9)
>4 cm	43 (17.1)	18 (41.9)	25 (58.1)	20	19 (95)	1 (5)
Encapsulated tumors, n (%)						
n	215	74 (34.4)	141 (65.6)	79	65 (82.3)	14 (17.7)
Yes	108 (50.2)	46 (42.6)	62 (57.4)	41	38 (92.7)	3 (7.3)
No	107 (49.8)	28 (26.2)	79 (73.8)	38	27 (71.1)	11 (28.9)
Invasion, n (%)						
Tumor capsule						
n	100	45 (45)	55 (55.0)	35	32 (91.4)	3 (8.6)
Yes	80 (80.0)	36 (45)	44 (55.0)	30	27 (90)	3 (10)
No	20 (20.0)	9 (45)	11 (55.0)	5	5 (100)	0 (0)
Vascular						
n	223	77 (34.5)	146 (65.5)	79	65 (82.3)	14 (17.7)
Present	80 (35.9)	28 (35)	52 (65.0)	37	30 (81.1)	7 (18.9)
Absent	143 (64.1)	49 (34.3)	94 (65.7)	42	35 (83.3)	7 (16.7)
Thyroid capsule						
n	222	77 (34.7)	145 (65.3)	79	64 (81)	15 (19)
Present	109 (49.1)	29 (26.6)	80 (73.4)	47	33 (70.2)	14 (29.8)
Absent	113 (50.9)	48 (42.5)	65 (57.5)	32	31 (96.9)	1 (3.1)
Extrathyroidal						
n	227	79 (34.8)	148 (65.2)	80	65 (81.2)	15 (18.8)
Present	84 (37.0)	23 (27.4)	61 (72.6)	38	27 (71.1)	11 (28.9)
Absent	143 (63.0)	56 (39.2)	87 (60.8)	42	38 (90.5)	4 (9.5)
Multifocality, n (%)						
n	228	79 (34.6)	149 (65.4)	80	65 (81.2)	15 (18.8)
Present	83 (36.4)	25 (30.1)	58 (69.9)	37	28 (75.7)	9 (24.3)
Absent	145(63.6)	54 (37.2)	91 (62.8)	43	37 (86)	6 (14)
Lymph node metastases, n (%)						
n	257	88 (34.2)	169 (65.8)	97	80 (82.5)	17 (17.5)
Present	93 (36.2)	34 (36.6)	59 (63.4)	60	46 (76.7)	14 (23.3)
Absent	164 (63.8)	54 (32.9)	110 (67.1)	37	34 (91.9)	3 (8.1)

Table 2 (continued)

Clinicopathological characteristics	DRP1 IRS score			S616-p-DRP1 IRS score		
	Total sample ($n = 259$, 100%)	Negative[a] ($n = 90$, 34.7%)	Positive[b] ($n = 169$, 65.3%)	Total sample ($n = 98$)	Negative[a] ($n = 81$, 82.7%)	Positive[b] ($n = 17$, 17.3%)
Distant metastases, *n* (%)						
n	258	90 (34.9)	168 (65.1)	97	97 (89.7)	10 (10.3)
Present	35 (13.6)	16 (45.7)	19 (54.3)	16	14 (87.5)	2 (12.5)
Absent	223 (86.4)	74 (33.2)	149 (66.8)	81	73 (90.1)	8 (9.9)
Molecular diagnosis, *n* (%)						
$BRAF^{V600E}$ mutation						
n	215	75 (34.9)	140 (65.1)	76	59 (77.6)	17 (22.4)
Positive	88 (40.9)	27 (30.7)	61 (69.3)	36	30 (83.3)	6 (16.7)
Negative	127 (59.1)	48 (37.8)	79 (62.2)	40	29 (72.5)	11 (27.5)
TERT promotor mutation						
n	187	68 (36.4)	119 (63.6)	66	50 (75.8)	16 (24.2)
Positive	23 (12.3)	11 (47.8)	12 (52.2)	9	8 (88.9)	1 (11.1)
Negative	164 (87.7)	57 (34.8)	107 (65.2)	57	42 (73.7)	15 (26.3)
Radioiodine treatment, *n* (%)						
Yes	219 (84.6)	73 (33.3)	146 (66.7)	83	66 (79.5)	17 (20.5)
No	40 (15.4)	17 (42.5)	23 (57.5)	15	15 (100)	0 (0)
No. of doses, *n* (%)						
n	219	73 (33.3)	146 (66.7)	82	65 (79.3)	17 (20.7)
1	140 (63.9)	39 (27.9)	101 (72.1)	44	36 (81.8)	8 (18.2)
≥2	79 (36.1)	34 (43)	45 (57.0)	38	29 (76.3)	9 (23.7)
Cumulative iodine dosage,						
n	241	84 (34.9)	157 (65.1)	91	74 (81.3)	17 (18.7)
mean ± SD, mCi	195.1 ± 235.1	230.0 ± 285.5	176.5 ± 201.6	223.9 ± 230.1	202.5 ± 217.7	317.4 ± 265.0
Disease-free status at the end of follow-up, *n* (%)						
n	236	78 (33.1)	158 (66.9)	90	73 (81.1)	17 (18.9)
Yes	168 (71.2)	50 (29.8)	118 (70.2)	57	47 (82.5)	10 (17.5)
No	68 (28.8)	28 (41.2)	40 (58.8)	33	26 (78.8)	7 (21.2)
Follow-up time, mean ± SD, years	8.6 ± 6.4	9.4 ± 6.5	8.2 ± 6.4	8.1 ± 6.0	6.6 ± 6.0	8.8 ± 5.1
Overall mortality	17 (6.6)	9 (52.9)	8 (47.1)	9	8 (88.9)	1 (11.1)
Disease-related mortality	9 (3.5)	3 (33.3)	6 (66.7)	5	5 (100)	0 (0)

BRAF B-Raf proto-oncogene, *DRP1* dynamin-related protein 1, *FTC* follicular thyroid carcinoma, *FVPTC* follicular variant of papillary thyroid carcinoma, *IRS* immune-reactive staining, *mCi* millicurie, *n* number of cases, *PDTC* poorly differentiated thyroid carcinoma, *Pos* positive, *PTC* papillary thyroid carcinoma, *SD* standard deviation, *S616-p-DRP1* serine 616-phosphorylated DRP1, *TERT* telomerase reverse transcriptase, *TNM* tumor, node, and metastasis

[a]Negative DRP1 expression was defined as IRS < 6

[b]Positive DRP1 expression was defined as IRS ≥ 6

S616-p-DRP1 expression according to clinicopathological characteristics

Patients who were less than 45 years presented a significantly higher S616-p-DRP1-positive expression rate vs. those who were 45 years or more (28.2 vs. 10.2%; $P = 0.013$).

The rate of S616-p-DRP1 positivity was 12.1, 0.0, and 16.7% for PTC, FTC, and PDTC, respectively (Table 2). Surprisingly, and unlike what was seen for DRP1 (Fig. 2e), none of the oncocytic variant cases was positive for S616-p-DRP1 expression (0.0 vs. 26.2%; $P = 0.028$) (Figs. 2f and 5a).

Tumors with infiltrative and mixed infiltrative/expansive margin growth patterns presented a significantly higher proportion of S616-p-DRP1-positive cases when compared with tumors with expansive margins (88.9 vs. 11.1%; $P = 0.033$) (Fig. 2c, d, showing high expression of S616-p-DRP1 and low expression of DRP1 in a PTC with infiltrative margins, and Fig. 5b). The same trend was observed for tumors with thyroid capsule invasion (29.8 vs. 3.1%; $P = 0.043$) (Fig. 5c). There were also more S616-p-DRP1-positive cases in patients with lymph node metastases (23.3 vs. 8.1%; $P = 0.012$) (Fig. 5d).

A statistically significant higher mean radioiodine cumulative dose was observed in patients who were positive for S616-p-DRP1 (317.4 ± 265.0 mCi vs. 202.5 ± 217.7 mCi; $P = 0.038$).

Interestingly, all five cases of patients who died due to TC were negative for S616-p-DRP1, and 14/16 cases of

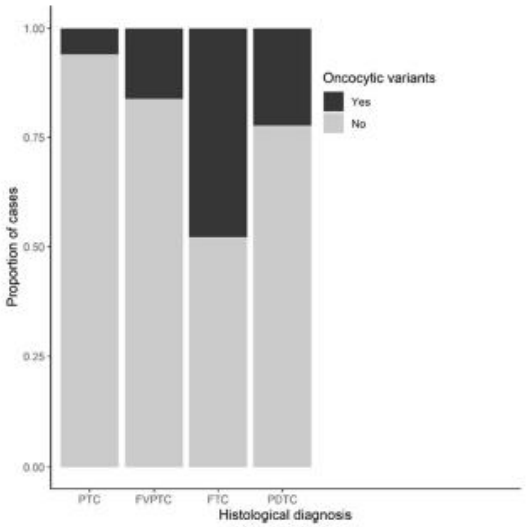
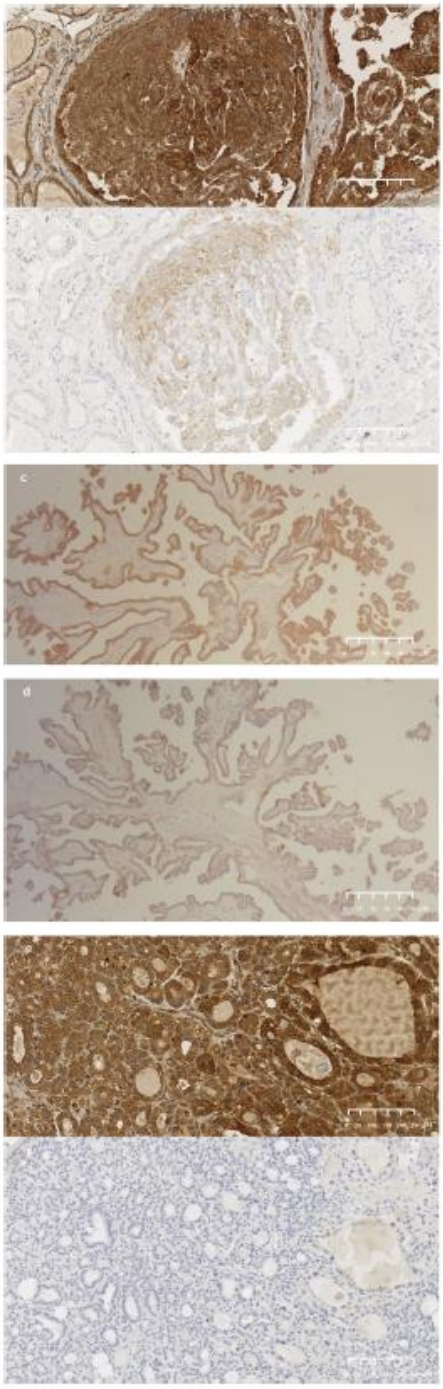


Fig. 1 Distribution of the histological type stratified by the presence or absence of oncocytic variants in the 259 TC cases. Black bars (yes) represent the reported proportion of oncocytic variant tumors in each histological type of TC. FTC follicular thyroid carcinoma, FVPTC follicular variant of papillary thyroid carcinoma, PDTC poorly differentiated thyroid carcinoma, PTC papillary thyroid carcinoma, TC thyroid cancer



patients who presented distant metastases were also negative for S616-p-DRP1 (Supplementary Table 2).

No significant association was found between S616-p-DRP1 expression and gender, tumor stage, tumor size, vascular invasion, extrathyroidal invasion, multifocality, distant metastases, $BRAF^{V600E}$ mutation, *TERT* promoter mutation, number of cycles, disease status at the end of follow-up, disease-related mortality, or overall mortality (Supplementary Table 1, and Supplementary Figs. 3 and 4).

Discussion

We studied the expression level of DRP1 and its active form, S616-p-DRP1, in a large series of FCDTC. Although there was no correlation between the expression of DRP1 and S616-p-DRP1, both were associated with locally invasive characteristics of the tumor. Aligned with reports in other tumor models [10, 21, 23, 42], the expression of DRP1 and S616-p-DRP1 was positively associated with locally invasive traits, as supported by a significantly higher number of positive tumors with thyroid capsule invasion. However, no significant association with poor prognosis factors was shown. Unlike DRP1, which showed positive in a large proportion of tumors (65.3%), S616-p-DRP1 was expressed in only 17.3% of those tested. This supports the correlative functional significance of S616-p-DRP1, as only a smaller fraction of DRP1 is relocated to the mitochondrial membrane as S616-p-DRP1 [43]. The higher proportion of S616-p-DRP1-positive cases was significantly associated

◀ **Fig. 2** Representative photomicrographs of the immunohistochemical analysis of DRP1 and S616-p-DRP1 staining in thyroid cancers. **a** Typical DRP1 expression is cytoplasmic without nuclear expression in a cPTC case with IRS score 9, ×20 magnification; **b** Typical S616-p-DRP1 expression is cytoplasmic without nuclear expression in a cPTC case with IRS score 2, ×20 magnification; **c** DRP1 expression pattern of a PTC case with IRS score 2, ×4 magnification; **d** S616-p-DRP1 expression pattern of the same PTC case as (**c**) with IRS score 12, ×4 magnification. **e** Oncocytic cells with high expression of DRP1 as assessed by an IRS score = 12, ×20 magnification; **f** Oncocytic cells with low expression of S616-p-DRP1 as assessed by an IRS score = 0, ×20 magnification. cPTC classical papillary thyroid carcinoma, DRP1 dynamin-related protein 1, IRS immune-reactive staining, PTC papillary thyroid carcinoma, S616-p-DRP1 serine 616-phosphorylated DRP1, TC thyroid cancer

with infiltrative margins, thyroid capsule invasion, and lymph node invasion, in line with previous reports suggesting a role of S616-p-DRP1 in tumor invasiveness in breast and lung cancer [21, 23].

"Aggressive variants" of TC, which in our analysis also included PDTC, were associated with a significantly lower proportion of DRP1-positive expression, in comparison with DTC. It is tempting to hypothesize that tumors with lower DRP1 expression might reflect the phenotypic expression of less differentiated tumors of most "aggressive variants". In agreement with this is the higher expression of S616-p-DRP1 in younger patients, which probably reflects

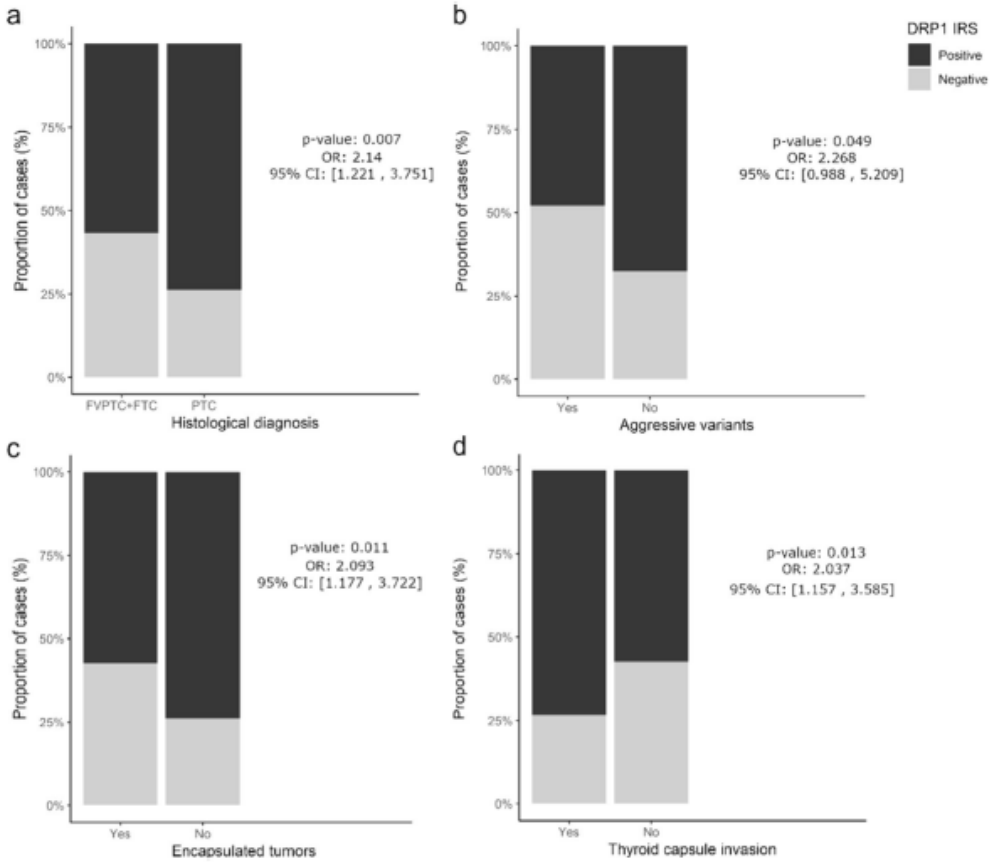


Fig. 3 Expression of DRP1 in (**a**) classic PTC cases vs. combined FVPTC and FTC cases; **b** more aggressive histologies—as defined in "Material and methods" section—vs. less aggressive histologies—differentiated TC (DTC) excluding "aggressive variants"; **c** encapsulated vs. non-encapsulated tumors; **d** tumors with vs. tumors without thyroid capsule invasion. Poor prognosis variants of PTC (e.g., solid/trabecular, diffuse sclerosing, tall cell, or columnar cell) were excluded from analysis (**a**). Black bars represent positive DRP1 expression (defined as IRS ≥ 6) and gray bars represent negative DRP1 expression (defined as IRS < 6). CI confidence interval, DRP1 dynamin-related protein 1, FTC follicular thyroid carcinoma, FVPTC follicular variant of papillary thyroid carcinoma, IRS immune-reactive staining, OR odds ratio, PTC papillary thyroid carcinoma

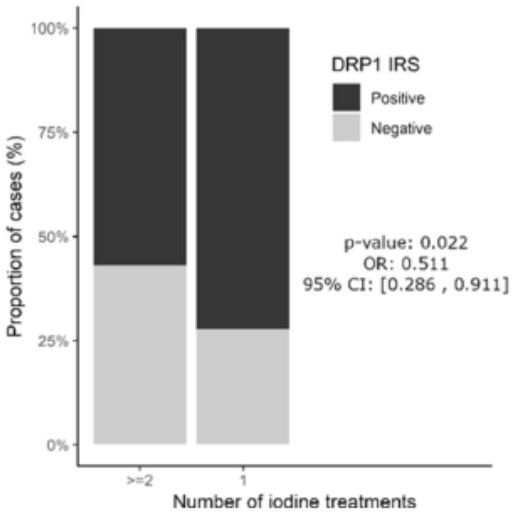


Fig. 4 Expression of DRP1 based on the number of radioiodine treatments. Black bars represent positive DRP1 expression (defined as IRS ≥ 6) and gray bars represent negative DRP1 expression (defined as IRS < 6). CI confidence interval, DRP1 dynamin-related protein 1, OR odds ratio

the phenotype of a self-regulated replicative tumor with a higher level of differentiation. This would not only resonate with the better prognosis of this patients' age group but also explain the apparent better response to the radioiodine therapy of these tumors, a concept that has been defended in other tumor models [20, 25].

Another point in favor of a link between higher DRP1 expression/activity and FCDTC differentiation is the significant association between lower expression of DRP1 and a higher number of radioiodine treatments. Interestingly, a statistically significant association between higher S616-p-DRP1 positive expression and a higher mean cumulative radioiodine dosage was observed. It is reasonable to admit that patients with locally invasive disease, including lymph node metastases, may have been treated with higher radioiodine dosages, even if the number of radioiodine treatments has not significantly differed in the overall patient population tested. In this respect, the apparent contradictory results between radioiodine treatment intensity observed for total DRP1 and S616-p-DRP1 expression could be justified.

We did not find any association between DRP1 or S616-p-DRP1 expression and distant metastases. Although the number of patients who presented distant metastases whose primary tumors were tested for S616-p-DRP1 was low, it is still noteworthy that 14 out of 16 were negative for S616-p-DRP1. Together with the fact that S616-p-DRP1 expression was associated with lymph node metastases, these data reinforce the idea that local and distant metastases have different molecular signatures in FCDTC, a concept that we discussed in a previous study of our group [44]. On the other hand, it is also possible that the lower S616-p-DRP1 expression represents a phenotype of cells less metabolically adapted to higher oxygen concentrations, and thereby less prone to blood-borne metastization. Indeed, the association between the loss of DRP1 with impaired glycolytic flux and the loss of mitochondrial metabolic function has already been described [45].

In FCDTC, only a relatively small number of patients will eventually die from the disease. This observation turns it difficult to relate DRP1 expression with a long-term clinical outcome. Nevertheless, our results showing a trend towards lower S616-p-DRP1 positivity in patients who died from the disease seem to be in line with the lower number of DRP1-positive cases in less differentiated tumors.

One of the most puzzling results of our study was the fact that S616-p-DRP1-positive cases were significantly lower in the 28 oncocytic variants of TC. We would expect that, given the higher mitochondrial biogenesis and deficient mitophagy processes described in these variants [46–54], S616-p-DRP1 would be more highly expressed in these tumors [55]. Our group has shown that DRP1 is overexpressed in oncocytic thyroid tumors, particularly HCC, suggesting that mitochondrial dynamics are dysregulated in Hürthle cells and that DRP1 might play a role in oncocytic tumorigenesis [10]. DRP1 is kept in an equilibrium between cytosolic and mitochondrial compartments [43, 56]. Recent data suggest that mitochondrial DRP1 may account for 40–50% of the overall DRP1 cell population [43, 56]. Mitochondrial fission is a complex process, which is dependent on the right amount and proper functioning of other dynamin-related proteins, posttranslational modifications, and also on the mitochondria lipid cardiolipin [57]. It is, therefore, likely that the ultrastructurally defective oncocytic mitochondria in an established tumor may lack the molecularly fit machinery needed for DRP1 oligomers to assemble in bigger helical-like structures, as described recently [43]. Under this hypothesis, S616-p-DRP1 could be highly expressed in the early stages of tumorigenesis in an attempt to compensate for the deficient mitochondria, but once the tumor reaches the established oncocytic phenotype, the opposite phenomenon is observed. The fact that no difference between DRP1 expression in oncocytic vs. non-oncocytic variants was found supports a posttranslational regulation of DRP1. The assessment of differentiation markers, such as NIS expression and iodine cell uptake, and their crosstalk with DRP1 as a key effector of mitochondrial bioenergetics and dynamic, could shed a light into the potential mechanisms of radioiodine resistance described in oncocytic thyroid tumors. The lower S616-p-DRP1 expression observed in tumors with oncocytic morphology could also explain, at least partially, why these tumors' cells are less prone to cell death. Of note, we found the same pattern

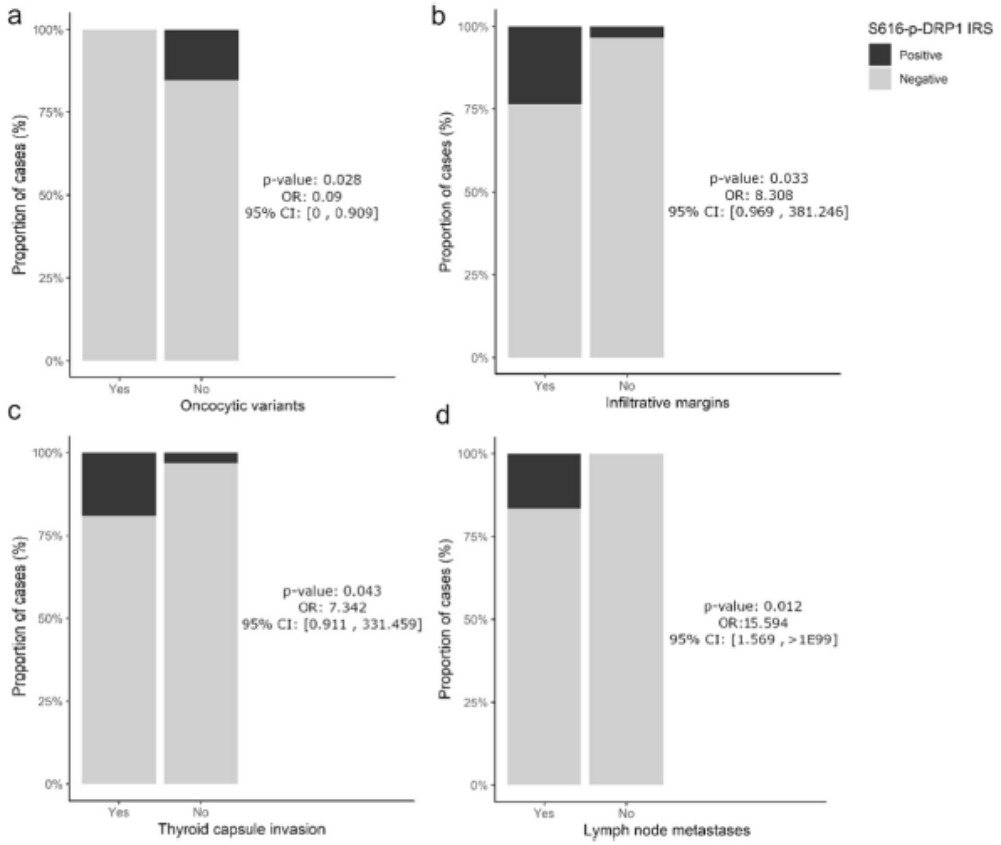


Fig. 5 Expression of S616-p-DRP1 in (**a**) TC cases by oncocytic vs. non-oncocytic carcinoma variants; **b** tumors with infiltrative margins vs. tumors with expansive margins; **c** tumors with vs. tumors without thyroid capsule invasion; **d** tumors with vs. tumors without lymph node metastases. Black bars represent positive DRP1 expression (defined as IRS ≥ 6) and gray bars represent negative DRP1 expression (defined as IRS < 6). CI confidence interval, IRS immunereactive staining, OR odds ratio, S616-p-DRP1 serine 616-phosphorylated DRP1

in a renal oncocytoma series (unpublished data). Whether this also explains radioiodine resistance itself is a question that deserves further study. Interestingly, when we treated the oncocytic TC cell line XTC.1 with a putative DRP1 inhibitor, Mdivi-1, which was shown to have anti-tumoral effects in various tumor models [10, 20, 22, 23, 42, 58], it was less sensitive to apoptosis when compared with other non-oncocytic TC lines (data not shown), highlighting the innate resistance to cell death of these tumors. In the future, mechanistic studies should address the relationship between DRP1, MAPK activity, and iodine uptake in depth, aiming at redifferentiating radioiodine-resistant tumors.

We herewith hypothesize that positive expression of S616-p-DRP1 can be used as a marker of infiltrative, locally invasive tumors, and lymph node metastases. Under this assumption, the assessment of S616-p-DRP1 expression as a candidate biomarker to be used in combination with the already established prognostic clinico-pathologic factors for pre and postoperative TC risk assessment in a larger TC patients sample should be further explored. Due to its association with locally invasive traits and lymph node mestastases, the IHC evaluation of S616-p-DRP1 in cytology, if feasible, could be of added value when deciding the extent of surgery to be performed. Furthermore, the opposing edges of S616-p-DRP1 expression spectrum should be evaluated and its biological significance validated through mechanistic work, given the apparently paradoxical negative expression in patients with poor outcomes. To this point, the fact that the oncocytic TC variants presented the same S616-p-DRP1 expression pattern as TC with distant metastases leads us to hypothesize that S616-p-DRP1 could be one of the molecular linking pieces between two phenomenona not seldomly observed in these two clinical entities—radioiodine and programmed cell death resistance [59].

Our study is limited by its retrospective nature, which translated in limited missing data and pathological and

staging classification systems used. However, this did not impact the results and our conclusions, particularly regarding the association with invasive disease. We have tested eight different definitions of DRP1- and S616-p-DRP1 positivity based on various IRS and intensity thresholds, and all have showed the same trends herewith reported (data not shown). Our results should be prospectively validated, and extended to further genotypes, including *RAS*- and *TP53*-mutated tumors. The validation of S616-p-DRP1 as prognostic factor also requires a prospectively proven significant association with hard endpoints, which is difficult to achieve given the relatively small number of patients who die due to TC.

In conclusion, both DRP1 and its activated form, S616-p-DRP1, are associated with locally invasive traits in TC. However, S616-p-DRP1 is likely a better candidate to predict locally invasive behavior of tumors, given its significant association with lymph node metastases. Therefore, validation of S616-p-DRP1 IHC assays and definition of expression scores with a clinical significance (e.g., positive vs. negative or high vs. low) should be prospectively addressed. We think that mechanistic studies should be performed exploring treatment strategies based on modeling DRP1 and MAPK activity in various TC models. This may pave the way to further tailor treatment strategies for patients who have persistent disease, become refractory to iodine treatment, and will eventually die from distant metastization—where the unmet medical need exists.

Data availability

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Consent to participate Samples were collected upon written informed consent of the patients, or their guardians, in case of patients under 18 years.

Ethics approval This work was approved by the Ethic Committee for Health (CES) of the Hospital Center of São João (CHSJ)/Faculty of Medicine of the University of Porto (FMUP) (CES 137 284–13) and by the Ethic Committee of the Faculty of Medicine of the University of Coimbra (n° 1309).

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Oncocytic thyroid neoplasms: from histology to molecular biology

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Abstract

The recent update of the 4th edition of the World Health Organization's *Classification of Tumors of Endocrine Organs* introduced important changes in the nomenclature of follicular-cell thyroid tumors, namely, regarding mitochondrion-rich neoplasms (In this review, for the practical purposes, the words Hürthle and oncocytic are synonymous in the field of thyroid pathology.) According to the last edition, oncocytic thyroid neoplasms, with follicular architecture and no typical nuclei of papillary carcinoma, – are now included in a separate group - the Hürthle cell neoplasms. Whenever thus categorized-while keeping oncocytic variant of papillary, medullary and poorly differentiated carcinoma-, a sort of tidal phenomenon has occurred about oncocytic tumors known for decades. Through this categorization, pathologists and researchers need to progress in the discussion about etiopathogenesis of oncocytic neoplasms (ONs). This review provides an attempt to balance the facts and doubts by questioning the recent changes based on what is known about oncocytic tumors.

Keywords genetics; Hürthle cell; molecular pathology; neoplasm; NIFTP; oncocytic; pathology; thyroid

Introduction

The most important alteration in the field of mitochondrion-rich/ONs in the last years was the re-identification of Hürthle cell neoplasms (Hürthle cell adenoma and Hürthle cell carcinoma). According to the 4th edition of the World Health Organization's *Classification of Tumors of Endocrine Organs*[1] (the 'blue book') such neoplasms are considered as a clinico-pathological entity that encompass benign and malignant neoplasms-not anymore variants of follicular adenoma (FA) and follicular carcinoma (FC). Together with the new classification, one has kept the presence of oncocytic variants of papillary/medullary/poorly differentiated carcinoma[1]

Histology

"Facts" about oncocytic (Hürthle cell) neoplasms

Few known neoplasms have been gaining so many names in their history as oncocytic thyroid neoplasms, reflecting the uncertainty about their nature: Askanazy cell tumor, oxyphilic cell tumor, Langhan's Struma, Baber cell tumor, Getzowa's Struma, Hürthle cell tumor, oncocytic cell tumor among others.[1] Although the terms "oncocyte" and "oncocytoma" were coined by Hamperl and Raffe in 1931 and 1932, respectively, curiosity about those cells among pathologists goes back to the Belle époque period.[2,3] In 1894, Karl Hürthle has actually described this peculiar morphology in parafollicular cells of dog thyroids.[4] In humans, this morphology had been described for the first time by Max Askanazy in 1898 in patients presenting with Graves' disease.[5] Despite this, the term "Hürthle" has persisted in the literature referring to thyrocytes as a synonym for full oncocytic morphology.

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In 2004, the 3th edition of WHO, endorsed the term "oncocytic tumors", instead of "Hürthle cell tumors" to clarify the terminology.[6] The tumors which were composed of more than 75% of oncocytes were classified as a variant under the title of papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC). At variance with previous approaches, the latest edition (4th) of WHO, creates a new tumor entity under the title of Hürthle cell tumors (HCTs).[1] What has caused this change after the last 13 years? What was/were the scientific fact(s) that have led to this current classification/categorization?

Although the scope of this review is the ONs of the thyroid gland, this phenotype is not specific neither to thyroid lesions, nor to tumors.[1] The term "Oncocyte/Hürthle" indicates the particular morphological appearance of a thyrocyte as a result of a huge increase in the amount of abnormally swelled mitochondria due to the mitochondrial dysfunction[7,8] and its description is quite clear: cells with a large, voluminous cytoplasm displaying a dense, granular, eosinophilic color with a distinct cell border and pink macronucleoli. The oncocytic morphology is not a "built in a day" phenomenon of the cell, but a step by step process *via* mitochondrial proliferation and morphological changes, since one has to accumulate thousands of mitochondria before the cells acquire an oncocytic/Hürthle cell phenotype.[7–9] The frequent occurrence in Hürthle cell neoplasms of ischemic foci with subsequent fibrosis either spontaneously or after FNA lead to prominent architectural alterations that mimics malignancy. (Virginia LiVolsi created the acronym WHAFFT (Worrisome Histologic Alterations Following Fine needle aspirations of Thyroid).[10]

Although HCTs were recognized by the 4th edition of the WHO 'blue book' of endocrine organs as a separate group of tumors, the discrepancy of mitochondrion-rich cell and oncocytic cell still unclear and have been gaining more importance lately.[1]

The characteristic staining of "oncocyte/Hürthle cell" lies behind a chemical attraction among cations and anions. The prominent eosinophilic appearance of the cytoplasm is related to the chemical interactions between the cationic organelles of the cell (e.g. mitochondria, lysosomes, endoplasmic reticulum, cytoflaments) with the anionic stain eosin. The cytoplasm reflects the number of cationic organelles in the cell by showing a spectrum from fully, dense, pink cytoplasm (oncocyte/Hürthle) to the less or incomplete oncocytic appearance. The latter has been named as "oncocytoid" or "mitochondrion-rich" by the pathologists and reflects the spectrum of morphological alterations.[8,11] However, neither at microscopy, nor at ultrastructural level, exists quantitative measurements for an exact distinction between "oncocytoid" cells from non-oncocytic counterpart. In a previous study, by our group, we attempted to analyze, based on molecular data, the demonstration of the progressive nature of the phenomenon[11] namely through the accumulation of mtDNA mutations, a genetic characteristic of these neoplasms. Indeed, the number of mutations in mtDNA, the pathogenesis of mutations and the mtDNA/nDNA (mitochondrial DNA/nuclear DNA) ratio, seem to increase from neoplasms in which there is a slight increase in the cytoplasm volume and of the eosinophilia, to neoplasms in which there is clearly an oncocytic phenotype.[9]

"Doubts" about oncocytic (Hürthle cell) neoplasms

The diagnostic criteria of follicular thyroid neoplasms and Hürthle cell thyroid neoplasms based on the WHO 2004 and 2017 were depicted into Figure 1[6,1]

At variance with follicular tumors, PTC, PDTC (poorly differentiated thyroid carcinoma) and MTC (medullary thyroid carcinoma) kept their variants for the oncocytic morphology, using the umbrella term of "oncocytic variant of…" The exact definition mentioned in the latest WHO classification[1] and based on of Tsybrovskyy *et al.*[12] article refers; *"Tumors with mitochondria-rich cells can be papillary (Tall cell variant, Warthin-like variant, Hobnail variant) or medullary and not be composed of Hürthle (oncocytic cells)"*. It naturally brings to our minds, how to differentiate mitochondrion-rich from oncocytic (Hürthle) cells? An ambiguous answer is advanced in the same paragraph: *"The cytoplasm of oncocytes is filled with mitochondria, with complete loss of cell polarity, to avoid confusion (which could have diagnostic and prognostic implications) these cells should be distinguished from "mitochondria-rich" cells that do not have complete loss of cell polarity and at the ultrastructural level have fewer mitochondria compared with Hürthle (oncocytic) cells"*. This explanation was referred to an elegant histochemistry-based article from 1960.[13]The issue remains unclarified, since hobnail variant of PTC (HV-PTC), some Tall-cell variant of PTC (TCV-PTC) and Warthin-like variant of PTC (WL-PTC) that have been described in the literature by its oncocytic morphology, regardless of performing histochemistry on all such "variants" (as well as all ONs)?.[14]There are a few, articles focusing on the separation of mitochondrion-rich vs ONs but there is no enough evidence to stress this necessity.[7,11]

Another interesting gap persists in another novel chapter of WHO 2017 classification ("other encapsulated follicular-patterned thyroid tumors") referring to a very low-grade malignant group of thyroid tumors, namely NIFTP (non-invasive follicular thyroid neoplasm with papillary-like nuclear features), FT-UMP (follicular tumor of uncertain malignant potential), WT-UMPs(well differentiated tumor of uncertain malignant potential).[1] In our daily practice, there are tumors that can be classified as NIFTP and UMPs displaying oncocytic characteristics, under the different cut-offs. Therefore, it would not be odd to question the existence of oncocytic "variants" of this particular type of tumors with low extremely low malignant potential (see Figures 2 and 3).

The studies about survival statistics and metastatic potential of ONs did not provide yet consistent data in the literature, namely regarding a prospective approach. Unfortunately, the answers remain unclear to address the putative clinical meaning of occurrence of oncocytic features in thyroid neoplasms that, *per se*, display a low malignant risk NIFTP, WT-UMP, FT-, WDC-, microPTC.

Molecular biology

This review is based as much as possible on Hürthle cell neoplasms. Despite the molecular features along the chapter being focused on Hürthle cell neoplasms, one cannot rule the possibility that some data have been included in studies from oncocytic variants of papillary carcinoma.

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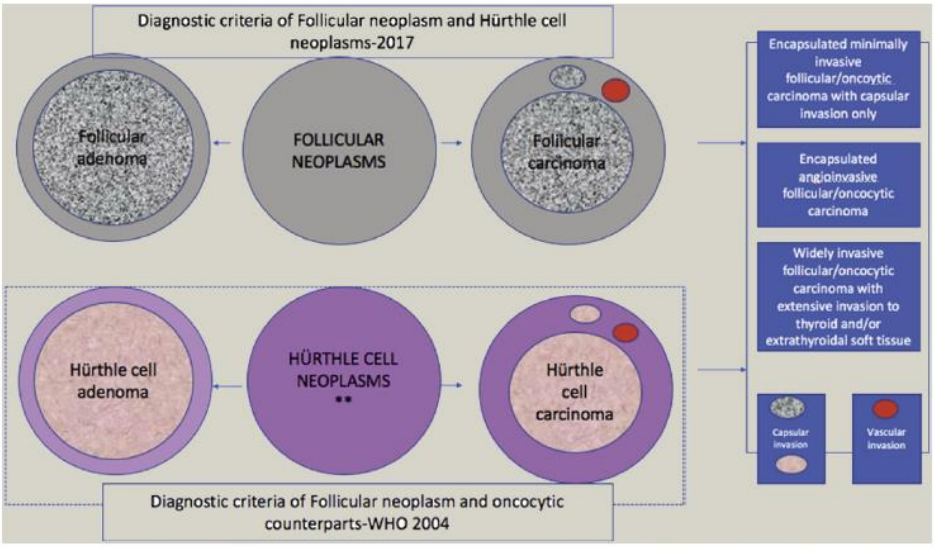

Figure 1 Diagnostic criteria of FNs and HNs (formerly "variants") based on the WHO 2004 and 2017. **There is similar problem in PTC with oncocytic morphology displaying a follicular/trabecular/solid growth pattern. In this context tumors lead to well-circumscribed tumors with or without a well-defined capsular invasion, and mainly, vascular invasion is the major criterion for diagnosing malignant neoplasm regardless of the nuclear features of PTC-like or not. FN, Follicular neoplasm; HN, Hürthle cell neoplasm.

Molecular biology is focused on genetic alterations of sporadic and familial (whenever adequate) forms of oncocytic thyroid neoplasms, including mtDNA alterations, chromosome copy number variations, nDNA mutations, microRNAs expression and DNA methylation pattern. For the daily practice of diagnostic pathology, we will concentrate on molecular pathology findings.

Mitochondrial DNA alterations

The characteristic alterations in ONs of the thyroid (and of other organs) is the high prevalence of mutations in mtDNA, that are scattered throughout the mitochondrial genome, including mitochondrial *tRNAs* and *rRNAs* genes, predominantly affecting genes encoding for complex I (CI) subunits of oxidative phosphorylation (OXPHOS) system, particularly ND1 and ND5 genes.[9,11,15,16] Most of the mutations lead to decreased expression or activity of several mitochondrial subunits of the OXPHOS system. Reported mutations in CI subunits tend to occur in conserved regions of the protein, important to their function, and tend to be more pathogenic.[11,15,16] These mutations suggest that, mitochondrial function is diminished (or even abolished), leading to a compensatory mechanism that triggers mitochondrial biogenesis in an attempt to restore the defective activity and ATP production, resulting in the acquisition of the oncocytic phenotype.[17] Indeed, increased expression of mitochondrial biogenesis

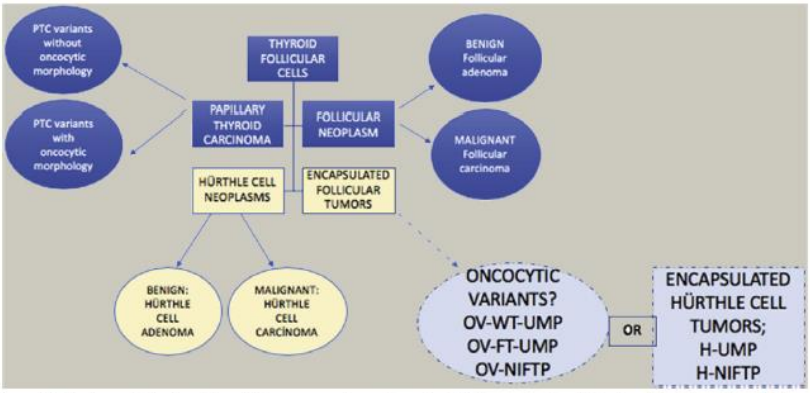

Figure 2 Classification of follicular cell derived thyroid tumors as based on the WHO endocrine 'blue book', 4th edition.[1] New changes are shown in yellow and questions in light blue.

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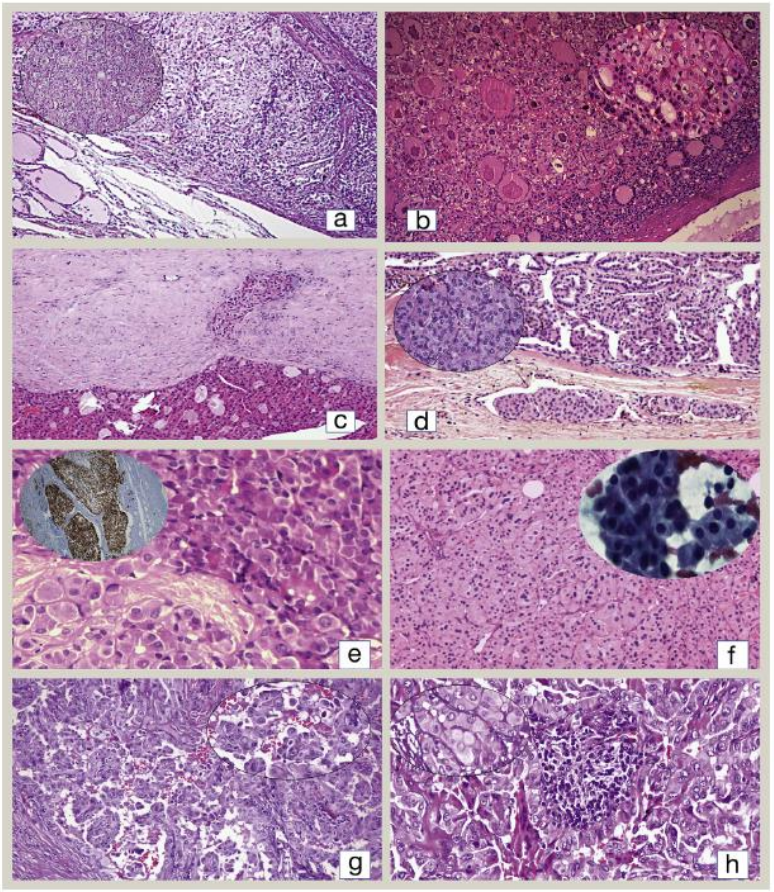

Figure 3 (a) A case of a "Hürthle cell adenoma" under medium power; inset, high power. (b) A case of a non-invasive follicular thyroid neoplasm with papillary-like nuclear features (with oncocytic characteristics) under medium power; inset, high power. (c) A case of a well-differentiated carcinoma, NOS (with oncocytic characteristics) under medium power. (d) A case of a Hürthle cell carcinoma under medium power; inset, high power. (e) A case of a oncocytic variant of medullary thyroid carcinoma under medium power; inset, calcitonin immunohistochemistry. (f) A case of a oncocytic variant of poorly differentiated carcinoma, based upon foci of extensive necrosis and extensive angioinvasion in a 9 cm tumor nodule under medium power; inset, high power. (g) A case of a hobnail variant of papillary thyroid carcinoma under medium power; inset, high power. (h) A case of a warthin-like papillary thyroid carcinoma under medium power; inset, high power.

proteins, has been described in oncocytic thyroid neoplasms in comparison with non- Ons.[18,19]

The 4977bp deletion in mtDNA called "common deletion" (CD; affecting mtDNA genes encoding for C I, C IV and ATPase subunits; as well as five tRNAs) is also frequent in ONs of thyroid and in other organs.[20,21] MtDNA CD is rarely present in non-oncocytic thyroid neoplasms, being present in all ONs.[21] Together with increased number of cells with abundant mitochondria, there is an increased number of mutated chromosomes in each cell. The process starts by heteroplasmy and evolves towards increasing the ratio of homoplasmy/heteroplasmy. In a full blown ON, the tumors display homoplasmy.[7] *ATPase 6* variants were associated with the occurrence of CD [21].

Along with somatic mtDNA mutations, mtDNA variants were described by Máximo *et al.* to be associated with increased risk of malignancy (oncocytic thyroid carcinomas).[21] Several germinative mtDNA gene variants in *CI, CIV and ATPase* genes were more frequent in patients harboring oncocytic carcinomas than in patients with non-oncocytic carcinomas[21]

Genetic predisposition to the occurrence of oncocytic neoplasms

The incidence of oncocytic thyroid neoplasms in a familiar panorama is higher in comparison to its incidence in a sporadic setting, being present in at least one subject in the majority of the families affected by thyroid non-medullary thyroid carcinoma (FNMTC). Presentation of the oncocytic phenotype in a familial setting has been reported in a non-syndromic context (in a background of FNMTC) as well as in a syndromic context with coexistence with other carcinomas in different organs (e.g. Birt

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–Hogg–Dubé syndrome, Cowden syndrome). Although some studies try to associate specific genes or common polymorphisms to an increased susceptibility of developing oncocytic thyroid neoplasms, there is a still high heterogeneity regarding the alterations were reported in the studies on record[22]

Canzian *et al*. previously mapped genetic events that increase susceptibility for oncocytic thyroid carcinomas to the "*Tumor with Cell Oxyphilia*" (*TCO*) locus at 19p13.2[22]. Maximo *et al.* in 2005, identified a germline mutation in *Gene associated with Retinoid-IFN-induced Mortality 19* (*GRIM-19*) gene, located at the *TCO* locus, in an individual showing an OV-PTC on a background of multiple Hürthle cell nodules, although no loss of heterozygosity in the tumor tissues was detected.[23] Thus, the question about the mechanism of action of this gene on thyroid ONs remains unclear.[24] Bonora *et al.* reported two mutations in Translocase of Inner Mitochondrial Membrane 44 gene (*TIMM44)* also located in *TCO* locus.[25] Although these mutations co-segregate with the TCO locus, other studies failed to detect such or other *TIMM44* variants thus rendering uncertain its role in familial HCT. In addition, Diquigiovanni *et al.* detected, in a family affected with FNMTC with oncocytic features, two novel heterozygous mutations in *Myosin IF* gene (*MYO1F*) also mapped in the TCO locus.[26] FRTL-5 thyroid rat cell line expressing these mutations revealed altered mitochondrial morphology and mitochondrial mass, suggesting that *MYO1F* may play a role in the establishment of a thyroid oncocytic phenotype.[26]

Additional genetic events, increasing susceptibility for oncocytic thyroid neoplasms, within a syndromic context were also described. Pradella *et al.* reported in a thyroid tumor of a patient with Cowden Syndrome (harboring a germline heterozygous *PTEN* mutation) a double heterozygosity in both *PTEN* and *Folliculin (FLCN*) genes.[27] This double loss was the only detectable tumorigenic hit in the thyroid ON and was suggested that this represents a new role of *PTEN/FLCN* double heterozygosity in syndromic oncocytic tumorigenesis.[28]

Lyu *et al* associated specific mtDNA haplogroups to an increased risk of thyroid oncocytic carcinomas.[28] Even though this increased risk does not fit within the concept of increased familiar risk, it represents an important step in general population predisposition. Haplogroups A and D5 showed an increased risk of ONs harboring mtDNA mutations in CI genes, (more than half in heteroplasmy) and in *rRNA* and *tRNA* regions.[28] The results enhance the insight towards the predisposition on ONs. Despite this positive result, it adds an increased complexity to the etiopathogenesis of ONs.

Genetic alterations of oncocytic neoplasms

RAS mutations: some studies pointed out different prevalence of RAS mutations in ONs compared to non-oncocytic counterparts (Table 1). Nikiforova reported *RAS* mutations in 8% and 11% of oncocytic adenomas and oncocytic carcinomas, respectively. In non-oncocytic tumors (48% and 52% in FTA (follicular thyroid adenoma) and FTC (follicular thyroid carcinoma), respectively), suggesting that ONs represent genetically a cluster of neoplasms different from their non-oncocytic counterparts.[29]

In line with this, *de Vries et al.* reported *RAS* mutations in only 6% of Hürthle cell (oncocytic) carcinomas (HCCs).[30]

Liu *et al.* found no *RAS* mutations in the 5 Hürthle cell adenoma (HCA) cases of their series, but found it in 8% in FTAs, and 38% in FTCs, in agreement with the previous reports.[31] In 2013, Ganly *et al.* reported *NRAS* mutations in 11% in HCC; out of those, one was minimally invasive HCC (MI-HCC) and two were widely invasive HCC (WI-HCC).[32] In 2018, the same group found a similar rate of *NRAS* mutation (9% - 2 MI-HCCs and 3 WI-HCCs) in a large HCC series. Combining *H–, K– and N-RAS* mutations, the overall percentage of RAS mutation increased to 15%.[15] From the reports on record, *RAS* mutations are less frequent in oncocytic than in non-oncocytic follicular neoplasms of the thyroid.

PAX8/PPARγ rearrangements: although scarce, *PAX8/PPARγ* rearrangements, in HCCs, were also described: between 27%, by de *Vries et al.*[30] and 2% by Gopal *et al*[16] Some other studies have not found *PAX8/PPARγ* rearrangements in the oncocytic cases of their series.[29,32] This shows that there is a wide variation of the prevalence of *PAX8/PPARγ* rearrangement (from 0 to 27%) in ONs, which may be related to the heterogeneity of the classifications. The percentage of *PAX8/PPARγ* rearrangement in HCTs is in line with the percentage of this rearrangement in FTCs and slightly higher than in FTAs (Table 1).

BRAF mutations: *BRAFV600E* mutations are prevalent in PTC,[33] but they are virtually absent in FTC and HCT.[32] Focusing on ONs, Trovisco *et al.* did not find BRAF mutations in HCA, HCC and FTC.[33] Although, Trovisco *et al.* reported *BRAF* mutations in 55% of oncocytic variant of PTC (OV-PTC) cases with conventional papillary thyroid carcinoma, whereas no mutation was observed in the eleven cases of OV-PTC with follicular growth pattern.[33] These results show the strong relation between *BRAF* mutations and the papillary pattern instead of the importance of the cell morphology.[34]

RET/PTC rearrangements: *RET/PTC* rearrangements are a common genetic alteration in PTCs, being also frequent in benign thyroid lesions (such as Hashimoto's thyroiditis) and ONs. Cheung *et al.* found *RET/PTC* rearrangements in 68% of the ONs included in its series: 100% of OV-PTCs, 54% of HCAs and 79% of HCCs[35]; de Vries *et al.* detected *RET/PTC* rearrangements in 33% of HCAs and in 38% of HCCs[30]; and Chiappetta *et al.* reported *RET/PTC* rearrangements in 57% in HCC and 58% in HCA.[36]

TERTp mutations: mutations in *TERT* promoter (*TERTp*) gene are frequent in a wide variety of human cancers, including thyroid tumors.[37,38] Our group detected no *TERTp* mutations in ONs,[37,39] whereas such mutations have been identified in ONs in other reports.[40]

Chindris *et al.*, reported *TERTp* mutations in 13% WI-HCC and in 15% MI-HCC,[40] while Landa *et al.* reported it in 24% WI-HCC, but none in MI-HCC.[41] Recently, Ganly *et al.*, noted *TERTp* mutations in 22% of the HCCs being 32% in WI-HCC and 5% in MI-HCC.[15] With the exception of the study of Chindris *et al*[40] It seems that less aggressive ONs tend to present lower prevalence of *TERTp* mutations (see Table 1). Regardless of this, ONs tend to harbor less *TERTp* mutations than the non-oncocytic counterparts, with frequencies varying from 5 to 10% in PTC and 10–25% in FTC (see Table 1)

Interestingly, mutations in other genes, related with telomere stabilization, were also detected in HCCs. Gopal *et al.* recently

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Genetic alterations in oncocytic compared to non-oncocytic neoplasms[1]

		Oncocytic neoplasms				Non-oncocytic neoplasms	
		HCA	HCC	OV-PTC	FTA	FTC	PTC
Genetic alterations	RAS	0%[31]–8%[29]	6%[30]–15%[15]	–	~30%[1]	30%–50%[1]	0–15%[1] (FV-PTC: 15%–35%)[1]
	BRAF	0%[33]	0%[15,30,33]	55%[33]	–	0%[1]	30–90%[1] (FV-PTC: 5%–25%)[1]
	PAX8/PPARγ	-	0%[29,32]–27%[30]	–	8%[1]	20%–30%[1]	–
	RET/PTC	33%[30]–57%[36]	38%[30]–79%[35]	100%[35]	–	–	5%–35%[1]
	TERTp	0%[37]	0%[37]–15%[40](MI-HCC)/32%[15] (WI-HCC)	0%[37]	–	10%–35%[1]	5%–25%[1]
	Other	–	TP53: 12%[16]–25%[42]	–	–	TP53: 0%[1]	TP53: 0%[1]
		–	PTEN: 41%[42]	–	PTEN: <5[1]	PTEN: < 10%[1]	PTEN: < 5%[1]

HCA, Hürthle cell adenoma; HCC, Hürthle cell carcinoma; MI, minimally invasive; WI, widely invasive; OV-PTC, oncocytic variant of papillary thyroid carcinoma; FTA, follicular thyroid adenoma; FTC, follicular thyroid carcinoma; PTC, papillary thyroid carcinoma; FV-PTC, follicular variant of papillary thyroid carcinoma.

Table 1

found *DAXX* mutations in 17% of the HCC in a mutually exclusive manner with *TERTp* mutations, which were found in 32% of the HCC cases.[16] Ganly *et al.* has also reported 3 HCC cases harboring *DAXX* mutations and one case with *ATRX* mutation.[15]

Other genetic alterations of tumor suppressor genes: alterations in other genes, such as *PTEN* and *TP53* were described in HCCs in 41% and 25%, respectively.[42] Recently, enrichment of mutations in genes from the TP53 signaling pathway were noted in ONs, after the Cancer Genome Atlas (TCGA) database.[11,34] Another recent report also observed *TP53* mutations in 12% HCC.[16] In non-oncocytic neoplasms, the frequency seems higher in ONs (see Table 1).

Copy number variations

In 2001, Erickson *et al.*[43] using fluorescence *in situ* hybridization (FISH) experiments, reported frequent chromosomal gains and losses in thyroid in benign and malignant oncocytic tumors. Chromosomal gains were mostly seen in chromosome 7, followed by chromosomes 5 and 12; whereas loss of chromosome 22 was most frequently observed. Loss of chromosome 22 was observed in 3 out of 11 samples from patients who died with HCC, suggesting it may be HCC prognostic marker.[43] In line with these findings it was recently reported higher chromosomal gains in the oncocytic group (including a large part of chromosome 7) and few chromosomal losses (that occur mainly on chromosome 22)[11]. Corver *et al.* reported that ten out of ten HCC cases had homozygosity in several chromosomes, and chromosome 7 retained heterozygosity in all neoplasms, suggesting it is important to favor the acquisition of oncocytic phenotype.[44] Overall, ONs tend to exhibit a "near-haploid" genotype,[45] but specific chromosomes tend to evade this by duplication.[15,16,44,45]

MicroRNAs in oncocytic neoplasms

In one of the first reports addressing the expression of microRNAs (miR) in ONs of thyroid published by Nikiforova *et al.*[46] miR-183, miR-197 and miR-339 were found to be overexpressed in HCC, while miR-31, miR-183 and miR-339 have higher overexpression in HCA, when compared to normal thyroid tissues. Cluster analysis of miR expression showed that clusters of Hürthle cell neoplasms are different from non-ONs.[46] These results reinforce the concept that Hürthle cell neoplasms have specific miR profile and are an independent class of thyroid tumors rather than a subgroup of conventional thyroid tumor histotypes.[6]

In study reported by Vriens *et al.*[47] differentially miR expression was observed between benign and malignant follicular thyroid tumors (including oncocytic and non-ONs). MiR-100, miR-125b, miR-138 and miR-768-3p were consistently downregulated in oncocytic adenomas *versus* oncocytic carcinomas. From those, both miR-138 and miR-768-3p were able to distinguish between benign and malignant oncocytic tumors (98% accuracy for both FNAB and tissues).[47]

When addressed the differences of miRs expression in PDTC, Dettmer *et al.* observed a higher expression of miR-221 and miR-885-5p in ONs than in non-ONs.[48] Oncocytic variant of PDTC had less expression of miR-125a-5p, miR-183-3p, miR-219-5p, miR-221 and miR-885-5p and more expression of miR-222 in comparison with HCC.

In another cohort of HCCs, including metastatic tumors, Petric *et al.* reported that miR-138 and miR-768-3p were consistently and significantly downregulated in all ONs compared to normal thyroid tissue.[49] Metastatic HCC patients had downregulation of miR-183, miR-221 and miR-885-5p compared to HCC patients without metastasis[49] (Table 2).

Methylation patterns in oncocytic neoplasms

Less attention has paid to DNA methylation status in oncocytic tumors. Perhaps the fact that no differences on global methylation of cytosine was found by Galusca *et al.* 2005 in HCA compared to FTA led to a lack of interest to new studies.[50] Still, Ganly *et al.* recently found in HCC that mutations in genes encoding for chromatin and DNA modifying enzymes (such as DNA or histone methyltransferases and demethylases, among others) are frequent: 59% out of 56 HCC studied cases.[15]

Changes in the methylation pattern of specific genes have been described in ONs, namely: *CDH1* promoter hypermethylation maybe associated to E-cadherin loss of expression in HCT[51]; *RUNX3* hypomethylation, in contrast with its hypermethylation in PTC cases[52] and *RASSF1* reduced methylation compared to FTC.[53]

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Summary of microRNAs comparative expression in oncocytic neoplasms

Comparative groups	microRNA	Regulation	Reference
HCA *vs* NT	miR-31, miR-183, miR-339	↑	46
HCC *vs* NT	miR-183, miR-197, miR-339	↑	46
HCA *vs* HCC	miR-100, miR-125b, miR-138, miR768-3p	↓	47
OV-PDTC *vs* PDTC	miR-221, miR-885-5p	↑	48
OV-PDTC *vs* HCC	miR-222	↑	48
	miR-125a-5p, miR-183-3p, miR-219-5p, miR-221, miR-885-5p	↓	48
HCC *vs* NT	miR-138, miR-768-3p	↓	49
HCC (metastatic *vs* non-metastatic)	miR-183, miR-221, miR-885-5p	↓	49

HCA, Hürthle cell adenoma; HCC, Hürthle cell carcinoma; NT, normal thyroid tissue; PD, poorly differentiated thyroid carcinoma; OV-PDTC, oncocytic variant of PDTC.

Table 2

Gene expression patterns in oncocytic neoplasms

Microarray analysis of gene expression in oncocytic thyroid neoplasms (including adenomas and carcinomas, the last both in MI-HCC and WI-HCC) was performed by Ganly *et al.* Using a clustering analysis, the authors observed that HA were more similar to MI-HCC, while WI-HCC were more distant from them, with few exceptions.[32] Analysis of gene expression enriched pathways demonstrated that β-catenin and vascular invasion pathway, as well as genes from pathways related to mTOR, were associated with WI-HCC in comparison to the data obtained in HA and HCA. Of note, WI-HCC did not cluster with PTC, showing a marked distance on genetic programs from them.[32] Recently, a genomic report using RNA sequencing, demonstrated again that MI-HCC and WI-HCC tend to cluster at some extend separately, based mostly in different enrichment in genes related to EIF2, EIF4, and mTOR pathways and related to mitochondrial activity.[15]

Prognosis of patients with oncocytic neoplasms

There has been a wide controversy regarding prognosis of HCC and their non-oncocytic counterparts. We summarize the key studies published on this topic in Table 3, including the role of surgery and radio ablative iodine (RAI). When we assess all published data, it is hard to conclude that HCC have a poorer prognosis than non-oncocytic counterparts. If any trend can be drawn, it appears that HCC carry a similar prognosis to non-oncocytic tumors, particularly if we take into consideration prognosis variables such as tumor size and gender.[54–57] It is important to highlight that many of the studies which indicated that HCC may be clinically different from the conventional FTC - in the sense that the former are more able to metastasize to lymph nodes and are associated with a higher recurrence rate and tumor-related mortality -, were retrospective and, with very few exceptions, mostly based on single institutional experience over several decades, where diagnosis and management has certainly evolved.[58,59] Indeed, the recurrence rates observed in HCC can vary from 14 to 44%[40,56,60,61] which underpins the potential bias(es) of many of the analyses. Some authors suggested that being HCC more often diagnosed in older, usually male patients, may be the reason why such tumors have been considered as more aggressive alluding that the earlier studies have not been compared between HCC and FTC.[57]

It was reported that adequate surgery is the most important treatment variable influencing prognosis[62] and this also holds true for HCC.[54,63–65] RAI treatment, on the other hand, was not found associated with better cancer-specific survival in HCC patients,[65] although it has shown a survival benefit in a cohort of patients which excluded T1 N0 M0 patients.[66]

Given all these data (Table 3), and the relative importance of disease extent and age for HCC prognosis, it is not surprising that the staging through the widely used AJCC = International Union against Cancer (AJCC = UICC) classification system based on pTNM (tumor, nodes, and metastasis) and age is also recommended for HCC, as for all thyroid carcinomas.[73]

Treatment of patients with oncocytic neoplasms

The study by Haigh *et al.* had suggested that patients with HCC should be treated as patients with equivalent tumors regarding stage of non-HCC.[57] The management of HCC is similar to that of FTC, acknowledging however two particular characteristics: metastatic HCC seem to be less prone to concentrating ^{131}I, and locoregional lymph nodes' involvement is more frequent, both having diagnostic and treatment implications,[74] and thereby the general consensus of HCC as a more aggressive form of follicular carcinoma. This latter fact has led to the standard surgical approach of bilateral thyroidectomy associated with compartmental lymphadenectomy.[6] The description of HCC clinical management follows the most recent ATA guidelines and National Comprehensive Cancer Network (NCCN) Thyroid Cancer guidelines.[74]

Total thyroidectomy is indicated in cases of invasive cancer and metastatic disease, and lobectomy is indicated in minimally invasive cancer without angioinvasion. If invasive cancer is identified with vascular invasion, completion of thyroidectomy should follow. RAI therapy should be considered in cases of gross extrathyroidal extension, when the primary tumor is more than 4 cm, when there is extensive vascular invasion or when postoperative unstimulated thyroglobulin (Tg) levels are over 5–10 ng/mL. RAI may be selectively considered if at least one of these conditions are present: primary tumors of 2–4 cm, minor tumor

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Summary of the key prognosis features of HCC

Study design	Tumor type	Sample size	Main prognosis findings	Reference
Retrospective, single-institution	HCC HCA	35 26	Aggressive surgical approach resulted in lower recurrence rate	Gundry *et al.*[54]
Retrospective, single-institution	HCC FTC	42 153	Assertive treatment of HCC provided a favorable outcome comparable to FTC	Khafif *et al.*[63]
Retrospective, population-based analysis (SEER database)	HCC FTC (matched) Excluded distant metastases	555 411	HCC and FTC had similar overall survival when controlling for gender and tumor size Increase in age, tumor size and male gender reduced survival in HCC patients	Bhattacharya *et al.*[55]
Retrospective, single-institution	HCC FTC	32 116	HCC and FTC had similar prognosis when controlling for age, gender, size, tumor extent and metastases. Recurrences were treated successfully in 33% of patients with FCT but were never cured in patients with HCC	Sanders *et al*[56]
Retrospective, single-institution	HCC	108	Long disease-specific survival observed in patients younger than 45 years, with no distant metastases and with no residual tumor after surgery	Petric *et al*[60]
Retrospective, single-institution	HCC FTC	72 156	Higher 10-years survival rates of HCC as compared to FTC patients, but unbalanced distant metastases and tumor size Tumor size and distant metastases were independent prognostic factors for survival	Besic et al.[67]
Retrospective, single-institution	HCC HCA	89 38	Older age and larger tumor size predicted for reduced survival Adjuvant ablation RAI provided a survival benefit; No survival benefit observed for the use of extensive surgery.	Lopez-Penabad *et al*[68]
Retrospective, single-institution	HCC FTC	172 673	Histology, local extension, thyroidectomy extension and RAI treatment had no effect on survival when age and gender are included; Older age, male gender, larger tumor size, lymph mode and distant metastases were associated with a higher mortality rate in both tumor types.	Haigh *et al*[57]
Retrospective, population-based analysis (SEER database) Retrospective, multi-institution	DTC Met-DTC HCC FTC PTC Mixed papillary/follicular carcinoma	58,515 1,291 24 108 103 471	FTC and HCC histotypes found to be independent predictors of poorer survival after adjustment for age, gender and stage. HCC presented worse survival and disease-free intervals when compared with mixed papillary/follicular carcinoma, PTC and FTC.	Goffredo P *et al*[69] Samman *et al*[70]
Retrospective, population-based analysis (SEER database)	Hürthle cell microcarcinomas (mHCC) Follicular cell microcarcinomas (mFTC) Papilary microcarcinomas (mPTC)	193 371 22,174	Follicular and Hürthle cell microcarcinomas presented more often with distant metastases compared with mPTC, and these patients had a poorer survival.	Kuo EJ *et al*[71]
Retrospective, multi-institution	HCC FTC	80 483	HCC patients were significantly older and presented more lymphovascular invasion; No difference between recurrence rates.	Kim *et al*[72]

Retrospective single-institution	HCC	62	Extent of surgery was an independent factor for cause-specific survival; Lymph node status presence of metastases at diagnosis and tumor stage were independent predictors of disease-free survival.	Mills *et al.*[64]
Retrospective single-institution	HCC	239	Total thyroidectomy as the main treatment approach was a predictive factor for better cancer specific survival.	Oluic *et al*[65]

HCC: Hürthle cell carcinoma, HCA: Hürthle cell adenoma, DTC: Differentiated thyroid carcinoma, FTC: Follicular thyroid carcinoma, PTC: Papillary thyroid carcinoma.

Table 3

invasion, cervical lymph node involvement, post-operative unstimulated Tg levels <5 ng/mL, or microscopic positive margins. For all other cases RAI is not typically recommended. As a general guidance, RAI ablation should be considered in those cases where patient clinic-pathological variables predict a high risk of disease recurrence, distant metastases, or disease-specific-mortality. By definition, RAI is not indicated in intrathyroidal tumors <2 cm, where there is no vascular invasion, clinical N0, no detectable anti-TG antibodies and post-operative unstimulated Tg <1 ng/mL. Pre-treatment ^{123}I whole body diagnostic imaging under TSH stimulation may be considered before RAI ablation, based on pathology postoperative Tg values, intraoperative findings and imaging assessments. Patients should then be followed at 6 and 12 months, and annually, with a standard workup including physical examination, assessments of TSH, Tg and antithyroglobulin antibodies, and regular neck ultrasound. For those patients who may be at higher risk of recurrence - intermediate and high-risk - this surveillance may include TSH-stimulated Tg levels and diagnostic RAI whole body scan.[75] However, it is important to keep in mind that there is a high false-negative rate for this diagnostic imaging technique in HCC, between 33% and 93%, depending on the metastatic site.[40] Other imaging techniques may include CT scan, chest radiography, and ^{18}FDG-PET.[76–78] ^{18}FDG-PET can be useful in following patients with HCC since there is an avidity of oncocytes for ^{18}FDG, and it has proven to be practice changing in concrete HCC cases given its high sensitivity and specificity.

Systemic treatment should be considered for patients with iodine-refractory unresectable persistent, recurrent or metastatic disease. Systemic tyrosine kinase inhibitors, which are FDA and EMA approved, for the treatment of differentiated iodine-refractory, progressive, locally advanced or metastatic thyroid carcinoma include levantininb,[79], -a vascular endothelial growth factor (VEGF) receptors 1, 2, and 3, fibroblast growth factor (FGF) receptors 1 through 4, platelet-derived growth factor (PDGF) receptor α, RET, and KIT inhibitor-, and sorafenib,[80] (a VEGFR-2 and PDGFR inhibitor, also targeting Raf kinase, Fms-like tyrosine kinase-3 (Flt-3) and stem cell growth factor (c-Kit)-can be considered. Distant metastases if symptomatic and/or progressive, may be target for resection or local therapies such as EBRT, stereotactic body radiation therapy (SBRT) or IMRT.

Conclusion

This brief review aims to obtain as much as possible the recent molecular data in articulation with solid surgical pathology. Based upon this, our group point out answered questions together with open and untouched questions in a realistic frame. We need to be aware of the subjectivity in the past, and some arbitrariness in the present, in order to be, hopefully, clear and solid in the future of ONs. ◆

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