Carneiro *et al.*
Surgical and Experimental Pathology (2024) 7:14
https://doi.org/10.1186/s42047-024-00157-1

Surgical and Experimental Pathology

REVIEW

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Predictive biomarkers in thyroid cancer in the current molecular-morphology paradigm

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Abstract

Thyroid cancer is one of the most common types of cancer worldwide. It is a spectrum of different diseases, ranging from very indolent to lethal tumors. Differentiated Thyroid Carcinoma (DTC), the most common thyroid malignancy, has often an excellent prognosis, but some patients develop metastatic Radioiodine-Refractory disease (RAIR) that cannot be controlled locally. In this setting, and for patients with metastatic Medullary Thyroid Carcinoma (MTC) and Anaplastic Thyroid Carcinoma (ATC), systemic treatment with non-selective Multikinase Inhibitors (MKIs) is often employed to improve survival rates and quality of life. The molecular characterization of thyroid cancer showed that the main drivers of thyroid carcinogenesis not only correlate with morphological and clinical features but can be targeted by some modern and highly selective Kinase Inhibitors: vemurafenib and dabrafenib for carcinomas with *BRAF* V600E mutation, including Papillary Thyroid Carcinoma (PTC) and its subtypes; dabrafenib in association with the MEK1/2 inhibitor trametinib for *BRAF* V600E-mutant ATC; larotrectinib and entrectinib for thyroid carcinomas with *NTRK* fusions and selpercatinib and pralsetinib for MTC with *RET* point mutations and DTC with *RET*-fusions. Apart of those markers, Microsatellite Instability status (MSI), Tumor Mutation Burden (TMB) and PD1/PD-L1 assessment have been explored in thyroid tumors, although immunotherapy for ATC has shown only modest results. Herein, we present a comprehensive review of the most relevant molecular markers with predictive value in thyroid pathology.

Introduction

Thyroid cancer is ranked as the 9th most common type of cancer worldwide, with a burden of 586.202 cases in 2020 (Sung et al. 2021). Mostly in high-income countries, a substantial rise in the detection of small/subclinical nodules by more precise imaging techniques in association with Fine-Needle Aspiration (FNA) lead to a progressive increase of the incidence rates in the last decades (Siegel et al. 2023; Kim et al. 2020; Vaccarella et al. 2016). However, overscreening seems to be responsible for only half of those new cases, as trends of true rise of the incidence, including large and advanced-stage tumors, as well as aggressive variants, are seen in some countries (Kim et al. 2020; Kitahara and Sosa 2020).

Most patients with thyroid cancer have Differentiated Thyroid Carcinoma (DTC) and, among them, the majority has Papillary Thyroid Carcinoma (PTC), the most common endocrine malignancy (Song et al. 2023; Romei and Elisei 2012), which usually carries excellent prognosis with standard treatment modalities. However, some patients with DTC may present recurrent, locally advanced or metastatic Radioiodine-Refractory disease (RAIR). For this subgroup, and for patients with metastatic Medullary Thyroid Carcinoma (MTC) and Anaplastic Thyroid Carcinoma (ATC) survival rates are

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noticeably lower, and systemic therapy with targeted drugs may be employed (Cabanillas et al. 2019).

Systemic agents applied to block the activity of molecular pathways associated with thyroid malignancies can be chosen independently from specific targets or, they can be highly selective against certain genetic abnormalities that can be detected by a myriad of ancillary techniques in pathology practice, the so-called predictive biomarkers. In this review, the main predictive biomarkers currently assessed in thyroid cancer will be explored, along with their main biological characteristics, applications, detection methods and related therapeutic agents.

Overview of malignant tumors of the thyroid and correlation with main molecular abnormalities

Malignant tumors of the thyroid are mostly carcinomas and encompass a broad spectrum of entities, from indolent to incurable tumors with different cells-of-origin. DTC is derived from follicular cells and represent 85% of thyroid carcinomas. Seventy-five percent, 15% and less than 10% of DTCs are represented by PTC, Follicular Thyroid Carcinoma (FTC) and Oncocytic Thyroid Carcinoma (OTC), respectively (Cabanillas et al. 2019). Differentiated High-grade Thyroid Carcinoma (DHGTC) was recently included in the DTC category and has intermediate prognosis, equivalent to that of the Poorly Differentiated Thyroid Carcinoma (PDTC); together, they account for 1% to 6.7% of thyroid carcinomas (WHO Classification of Tumours Editorial Board 2022). Lastly, dedifferentiated thyroid carcinoma (ATC) represents 1% to 4% of cases of thyroid malignancies worldwide, regarded as the most aggressive and rarest type of follicular cell-derived tumors (Abe and Lam 2021). Less than 5% of thyroid carcinomas are MTCs, originated from parafollicular (C cells) of the neural crest; 30% of them are hereditary, related or not to Multiple Endocrine Neoplasia type 2 (MEN2). Much rarer entities are exemplified by thyroid carcinomas of salivary-gland type, tumors of thymic or embryonic origin and carcinomas of uncertain histogenesis, as the morular-cribriform carcinoma and the sclerosing mucoepidermoid carcinoma with eosinophilia (Cabanillas et al. 2019; WHO Classification of Tumours Editorial Board 2022; Gild et al. 2023).

The main molecular drivers of follicular-cell derived thyroid carcinomas are components of the MAPK-ERK pathway (*Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase*), a vital pathway implicated in many basic cellular functions: growth, differentiation, proliferation, transformation, motility and apoptosis. Among the 4 classic MAPKs described, MAPK-ERK is the most extensively studied so far, and its association with the development of various malignant and benign tumors is known since the 1990's (Zhang et al. 2002; Bahar et al. 2023). MAPK-ERK consists of a cascade of proteins capable of transduction and amplification of extracellular signals initiated after the interaction between membrane kinase receptors and their ligands, such as mitogens or cytokines, or even under stress conditions (ligand-independent activation). The signal transmission sequence begins after the receptor's dimerization/phosphorylation, which activates one of the membrane-based, homologous GTPases from the RAS family (H, K and NRAS). At this point, the inactive GDP-bound form of the RAS protein releases GDP to become an active GTP-bound form, able to recruit the next component of the pathway, a RAF protein. Then the following proteins MEK and ERK are successively activated until the signal reaches the nucleus, a process dependent on sequential phosphorylation of each component (Fig. 1) (Zhang et al. 2002; Bahar et al. 2023; Hernandez-Prera 2021).

Less commonly associated with follicular-cell derived carcinomas are some mutations in the PI3K-AKT pathway, some of them linked to anaplastic transformation; the *PAX8-PPARγ* fusion, seen in up to 35% of FTCs and in the Invasive Encapsulated Follicular Variant of PTC (IFVPTC) and mutations in *EIF1AX*, *PTEN*, *DICER1*, also met in follicular patterned tumors. MTC is predominantly associated with *RET* point mutations which ultimately leads to abnormal activation of MAPK and other pathways (Cabanillas et al. 2019; WHO Classification of Tumours Editorial Board 2022; Hernandez-Prera 2021; Al-Jundi et al. 2020).

The scheme proposed by the 5th Edition of the World Health Organization (WHO) Classification of Endocrine Neoplasms supports the observation that the main genetic abnormalities involving the MAPK-ERK pathway found in DTC correlate, at least in general, with histopathological and clinical features. So, two major molecular-morphologic subgroups of DTC can be identified: a *RAF*-like subgroup, with *BRAF* mutations (mostly V600E), comprised of classical PTC and its subtypes, with papillary architecture and typical PTC-like nuclear features, and a *RAS*-like subgroup, comprised of FTC and IFVPTC, the latter with less pronounced PTC-like nuclear features and similar clinical behavior to FTC. The Non-Invasive Follicular Thyroid neoplasm with Papillary-like nuclear features (NIFTP), a very low-risk tumor, despite its nuclear characteristics, has almost exclusive follicular pattern and a molecular profile that sets it closer to the *RAS*-like family. Two other less common groups are more related to *RAF*-like tumors: PTCs with *RET* fusions (so-called *RET*/PTC *genes*) and PTC with *NTRK* fusions, both discussed in more detail bellow (Fig. 2) (Romei and Elisei 2012; WHO Classification of Tumours Editorial Board

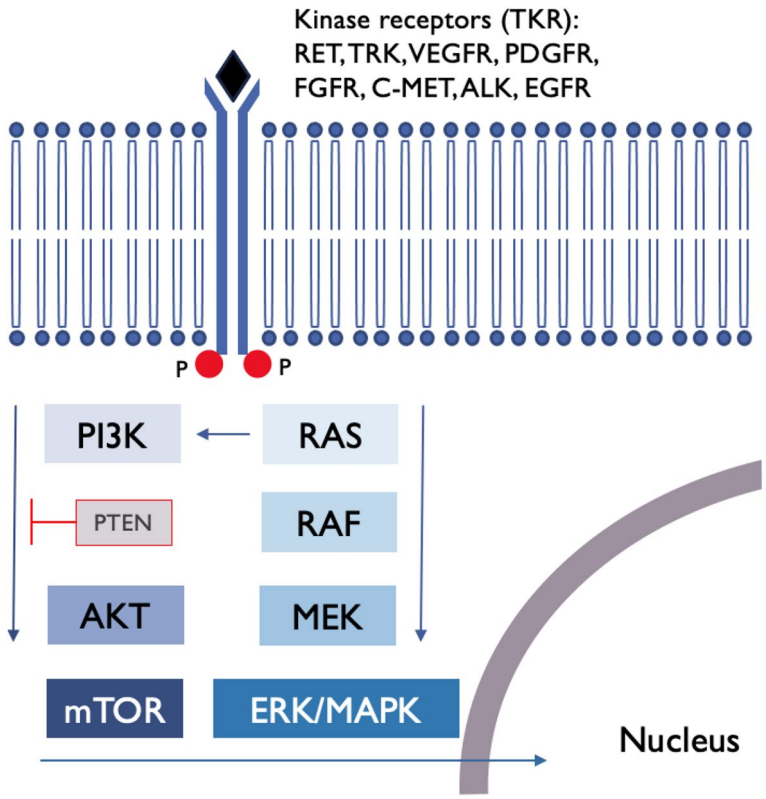

Fig. 1 Schematic representation of MAPK-ERK and PI3K-AKT pathways. Dimerization and phosphorylation of the various Kinase Receptors' intracytoplasmic Kinase domain triggers phosphorylation and activation of following components, which allows transmission of extracellular signals to the nucleus

2022; Chu et al. 2020; Baloch et al. 2022; Vodopivec and Hu 2022).

Predictive biomarkers in thyroid cancer

When and for whom

A biomarker is any objective measurement of a normal or pathological process, including response to clinical or any other therapeutic intervention. In the field of pathology, biomarkers related to cancer are usually assessed in tissue, blood or other biological fluids and they usually represent specific molecular aspects of the tumor biology. Didactically, biomarkers can be divided in 1) diagnostic, allowing accurate classification of the tumor type; 2) prognostic, which provide information about the tumor biological behavior and 3) predictive, defined as alterations able to estimate the level of response to a specific therapy. A single biomarker can be simultaneously diagnostic, prognostic and predictive. Although not new, predictive biomarkers are the foundation for personalized approach in the contemporary oncology practice, which dramatically changed survival and prognosis of patients with many types of cancer in the last decades (Passaro et al. 2024; La Thangue and Kerr 2011; da Cunha et al. 2021).

Patients with DTC have an overall Disease-Specific Survival (DSS) of more than 90% in 10 years when conventional treatment is implemented (i.e. surgery for removal of the primary tumor with or without regional lymph node dissection, followed by Radioactive Iodine Therapy (RAI)/hormone therapy in high-risk or metastatic disease. Nonetheless, local recurrence and distant metastasis, mostly from RAIR carcinomas, can occur in 20% and 10% of patients, respectively, the risk being higher in those with locally advanced disease, characterizing a group of patients with a significant shorter survival (Cabanillas et al. 2019; Al-Jundi et al. 2020; Cavalheiro et al. 2023; Haugen et al. 2016; Shen et al. 2024). On the other hand, ATC has invariably a fatal outcome, and the initial approach rarely includes surgery but rather systemic therapy (Cabanillas et al. 2019; WHO Classification of Tumours Editorial Board 2022; Al-Jundi et al. 2020; Haugen et al. 2016; Agosto Salgado et al. 2024; 12325_2020_Article_1391 n.d.). Prognosis of MTC is largely dependent on stage and other pathologic characteristics of the tumor. Lymph nodes and distant

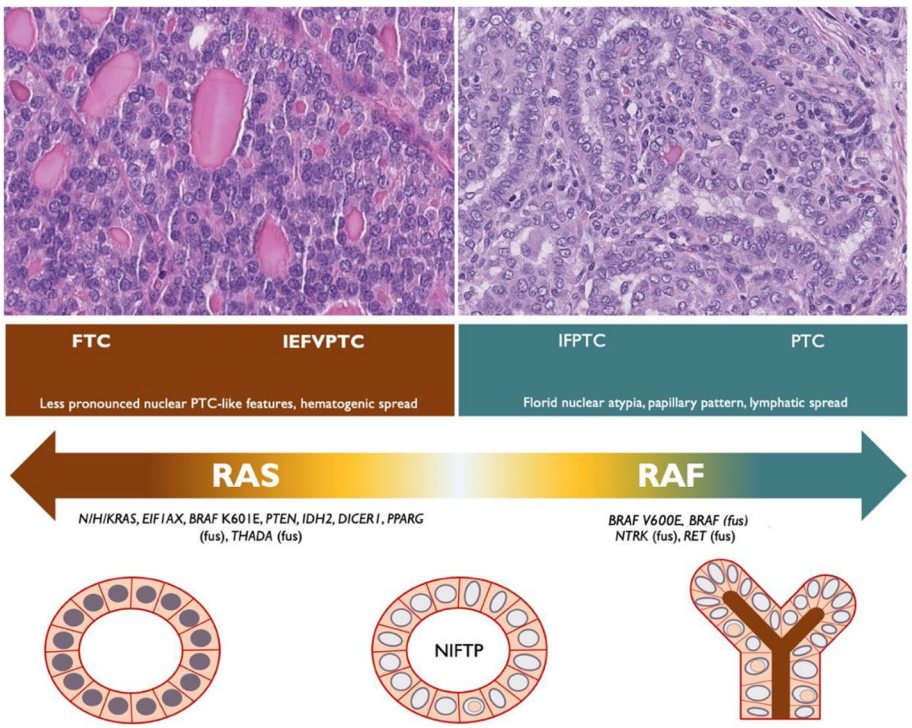

Fig. 2 Morphological and clinical differences between *RAF*-like and *RAS*-like tumors. While the *RAS*-like family is represented prototypically by follicular patterned tumors FTC and IVFTC, which have predominant hematogenic spread, *RAF*-like carcinomas show papillary architecture, typical PTC-like nuclear features and mostly lymphatic spread. NIFTP has PTC-like nuclear features but is closer to the RAS-like tumors at molecular level, sometimes harboring the *BRAF* K601E mutation. Images from the Archives of Anatomic Pathology Department, Rede D'OR São Luiz

metastasis are present in up to 70% and 15% of patients at diagnosis. Localized MTC can be managed surgically, and the 5-year survival for stages I-III is 93%. In contrast, patients with distant metastasis have a much lower 5-year survival of only 21%. Other factors that have prognostic value in MTC, such as the *RET* M918T mutation and the graduation according to the International Medullary Thyroid Carcinoma Grading System (IMTCGS), a two-tiered system which separates high- and low-grade MTC based on tumor necrosis, mitotic count and ki-67 index (WHO Classification of Tumours Editorial Board 2022; Gild et al. 2023; Agosto Salgado et al. 2024; Xu et al. 2021; Barletta et al. 2021).

Patients with metastatic RAIR DTC or MTC can be actively observed with successive measurements of biochemical markers and imaging when there are no

Table 1 Main targets, corresponding predicitive biomarkers, detection methods and therapeutic agents in the context of advanced, metastatic thyroid cancer

Target	Predictive Biomarker	Detection	Tumor type	Targeted drug
BRAF	*BRAF* V600E	IHC, RT-PCR, Sanger, NGS	RAIR PTC	Vemurafenib, Dabrafenib (redifferentiation)
BRAF + MEK	*BRAF* V600E	IHC, RT-PCR, Sanger, NGS	ATC	Dabrafenib + Trametinib[a]
NTRK A, B, C	*NTRK* fusions	IHC, FISH, NGS	Tumor agnostic	Larotrectinib[a], Entrectinib[a]
RET	*RET* Fusions	IHC, FISH, RT-PCR, NGS	PTC, PDTC, ATC, OC	Selpercatinib[a], Praseltinib[a]
RET	*RET* point mutation	IHC, FISH, RT-PCR, NGS	MTC	Selpercatinib[a], Praseltinib[a]
PD1	MSI-h, TMB-h, PD-L1 expression	IHC (MMR/PD-L1[b]), NGS	Tumor agnostic	Pembrolizumab[a] and other ICI

MSI-h Microsatellite Instability-High, *TMB-h* Tumor Mutation Burden-high, *IHC* Immunohistochemistry, *RT-PCR* Real-time Polymerase Chain Reaction, *NGS* Next-Generation Sequencing, *MMR* Mismatch Repair, *RAIR* Radioiodine-Refractory, *PTC* Papillary Thyroid Carcinoma, *ATC* Anaplastic Thyroid Carcinoma, *PDTC* Poorly Differentiated Thyroid Carcinoma, *OC* Oncocytic Carcinoma, *MTC* Medullary Thyroid Carcinoma, *ICI* Immune Checkpoint Inhibitors

[a] FDA-approved

[b] There is no cut-off validated for PD-L1 expression

symptoms or significant structural disease progression. In the RAIR DTC scenario, when there is progression with symptoms or increased risk of morbidity and mortality, local treatment of metastatic foci, both surgical and non-surgical, can be offered. Usually after failure of local control of metastatic DTC, as well as in the metastatic MTC and as first option in patients with ATC, drugs targeting the main drivers of thyroid tumorigenesis or immune checkpoint molecules can be applied, with promising results (Cabanillas et al. 2019; Al-Jundi et al. 2020; Agosto Salgado et al. 2024).

Targeted agents in thyroid carcinoma are mostly Tyrosine Kinase Inhibitors (TKIs) directed to MAPK-ERK pathway components or kinase receptors like RET, NTRK and ALK. The use of TKIs for treatment of malignant thyroid tumors dates to the 2010's, with the approval of lenvatinib and sorafenib for RAIR DTC and cabozantinib and vandetanib for advanced/metastatic MTC (Barletta et al. 2021a; Wells et al. 2012; Schlumberger et al. 2015; Elisei et al. 2013). All these drugs have an anti-angiogenic effect due to inhibition of Vascular Endothelial Growth Factor Receptor (VEGFR). However, they are non-selective Multikinase Inhibitors (MKIs), which means they can target multiple receptors and other components of the MAPK pathway. Despite their effectiveness, the lack of specificity is responsible for many important side effects such as hypertension, asthenia, weight loss, hand-foot-skin reaction, hypocalcemia, proteinuria, among others, which can determine treatment interruption or discontinuation (Shyam Sunder et al. 2023; Vodopivec and Hu 2022). They also can be applied regardless of the status of predictive biomarkers in tumors without an actionable abnormality, namely FTC, PDTC and ATC carrying *RAS* mutations and OC, which normally does not have altered genes typically seen in other thyroid carcinomas (WHO Classification of Tumours Editorial Board 2022; Al-Jundi et al. 2020).

Advances in molecular characterization of thyroid carcinomas in parallel with the development of more potent and specific TKIs brought about important predictive biomarkers in clinical and pathology practices, the most important of them to be explored in the following sections. Table 1 provides a synthesis of those biomarkers with their matching tumor types and FDA-approved drugs.

Selected predictive biomarkers in thyroid pathology

BRAF (murine sarcoma viral oncogene homolog B)

BRAF is one of the members of RAF Serine/Threonine Kinases family (composed of ARAF, BRAF and CRAF), a direct effector of RAS and an activator of MEK in the MAPK-ERK canonical pathway. The *BRAF* gene is one of the most frequently mutated genes in human cancer, with about 200 mutant alleles identified in different malignant tumors, making it an important oncogenic driver. Depending on the resulting amino acid sequence, *BRAF* mutations can have diverse effects on the functionality of the gene and also be a target for selective drugs (Bahar et al. 2023; Rangel-Pozzo 2020; Zaman et al. 2019; Fu et al. 2023; Scheffel et al. 2022; Dankner et al. 2018). In PTC, the most common *BRAF* abnormality is the point mutation T1799A, which switches a Thymine (T) for an Adenine (A) at nucleotide position 1799 in the exon 15 (*BRAF* T1799A), resulting in Valine's (V) replacement by a Glutamic Acid (E) at residue 600 of the BRAF protein (*BRAF* V600E) (Bahar et al. 2023; Rangel-Pozzo 2020; Zaman et al. 2019; Fu et al. 2023; Scheffel et al. 2022; Dankner et al. 2018).

BRAF V600E is found in 18% to more than 90% of PTCs depending on the subtype and accounts for 98–99% of *BRAF* alterations in PTC. The tall cell and hobnail PTC subtypes, both aggressive forms of papillary carcinoma associated with frequent recurrences, distant metastasis and general poor outcome, have some of the highest frequencies of this mutation (Fig. 3). *BRAF* V600E can also be present in DHGTC, 33% of PDTCs, 20–50% of ATCs and, more rarely, in up to 5% of OCs. Infrequently, BRAF fusions can be found in DTC in the adult population. Of note, *BRAF* K601E mutation is not associated with *RAF*-like neoplasms, but rather with the *RAS*-like subgroup, and is seen in some cases of NIFTP. Neither *BRAF* V600E nor *BRAF* fusions have been described in FTC, benign nodules or MTC (WHO Classification of Tumours Editorial Board 2022; Hernandez-Prera 2021; Al-Jundi et al. 2020; Rangel-Pozzo et al. 2020; Haroon Al Rasheed and Xu 2019; Zhang et al. 2023; Subbiah et al. 2017; Morandi et al. 2017; Teng et al. 2017; Alzahrani 2023; Landa et al. 2016; Cancer Genome Atlas Research Network 2014).

The presence of a *BRAF* mutation has been associated with adverse prognostic features in PTC, such as less-differentiated tumors, larger size (>2 cm), diminished response to RAI, risk of lymph node metastasis and recurrence (Rangel-Pozzo 2020; Haroon Al Rasheed and Xu 2019). This is a controversial topic, as other studies have questioned the real prognostic value of *BRAF* V600E by itself, considering the impact of many other clinicopathological features on patient's outcome and the presence of this mutation in a significant percentage of patients with small intrathyroidal carcinomas (Alzahrani 2023; Xing et al. 2013; Li et al. 2015; Wang et al. 2018).

Various techniques can be performed to detect *BRAF* V600E and other *BRAF* abnormalities in Formalin-fixed Paraffin-Embedded tissue (FFPE): Sanger sequencing, immunohistochemistry (IHC), Next-Generation Sequencing (NGS) and Polymerase Chain Reaction (PCR). Sanger sequencing is a traditional and commonly

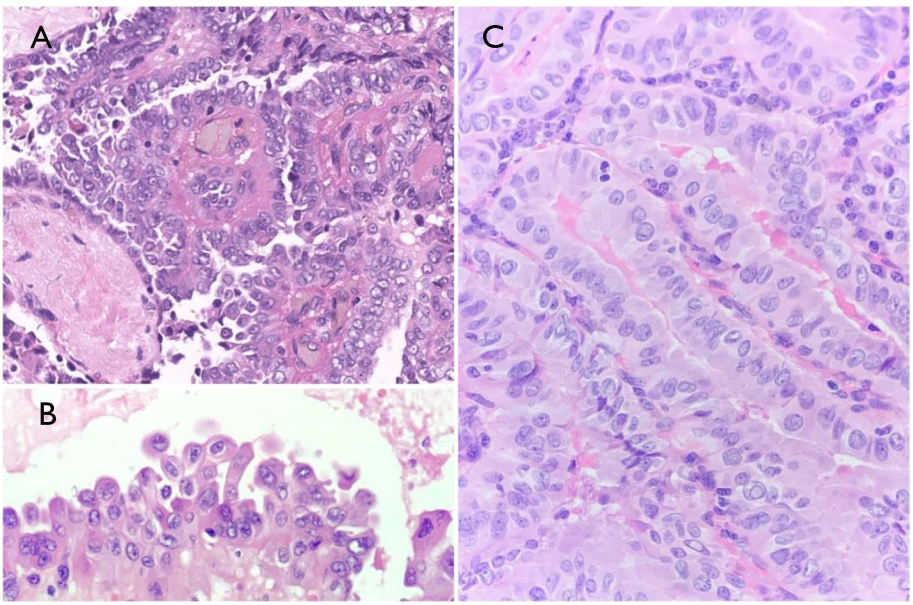

Fig. 3 Hobnail (**A**, **B**) and tall cell (**C**) are aggressive PTC subtypes with frequent *BRAF* V600E mutations. Images from the Archives of Anatomic Pathology Department, Rede D'OR São Luiz

used method in laboratories, but due to its relatively low sensitivity, detection of mutations requires a relatively large amount of tumor DNA. PCR, on the other hand, has greater sensitivity and can provide the quantification of the mutant allele. In one study, droplet digital PCR (ddPCR) detected *BRAF* V600E in 76.67% of PTC patients, in comparison to 55.83% by Sanger sequencing. Droplet dPCR showed sensitivity and specificity of 100% and 69.88%, respectively, and, unlike the Sanger technique, was able to detect mutated cases at a fractional abundance of < 5% (Rangel-Pozzo 2020; Haroon Al Rasheed and Xu 2019; Fu et al. 2021; Wang et al. 2019; Ylli 2019; Kouba et al. 2018).

NGS has high accuracy, simultaneously assessing *BRAF* and other mutually exclusive (targetable or not) cancer-associated genes. Along with PCR, it is feasible not only in FFPE, but in FNA and liquid biopsy specimens (Kouba et al. 2018; Ye et al. 2019). Immunohistochemistry (IHC) is another cost-effective alternative for indirect detection of *BRAF* mutations using the VE1 clone. It can be helpful when dealing with decalcified tissue with fragmented DNA which can make molecular testing unsuccessful. However, despite the good concordance between strong positive BRAF cytoplasmic staining and presence of *BRAF* mutation, results are uncertain in the cases of moderate or faint staining (Abd Elmageed et al. 2017; Bourhis et al. 2019).

Given the association of *BRAF* V600E with the diminished ability to incorporate radioactive iodine by tumor cells, causing treatment failure or recurrences in metastatic RAIR PTC, additional modalities of systemic treatment are often needed (Xing et al. 2013; Brose et al. 2016; Dunn et al. 2019; Falchook et al. 2015; Rothenberg et al. 2015). Vemurafenib is a selective TKI targeting mutated *BRAF* with antitumor activity and redifferentiation effect in patients with progressive RAIR *BRAF* V600E-mutant PTC (Brose et al. 2016; Dunn et al. 2019). In one study, redifferentiation in *BRAF* V600E-mutant RAIR-PTC was achieved in 4 of 10 patients who responded to RAI after therapy with vemurafenib, resulting in tumor regression after 6 months. Further analysis demonstrated that inhibition of the MAPK pathway by vemurafenib was associated with increased thyroid gene expression and RAI uptake (Dunn et al. 2019).

Similarly, dabrafenib, another highly selective and potent BRAF inhibitor, can induce radioactive iodine uptake in up to 60% of patients with unresectable or metastatic *BRAF* V600E-mutant RAIR PTCs (Zhang et al. 2023; Falchook et al. 2015; Rothenberg et al. 2015). Retrospective and prospective studies on BRAF inhibitors dabrafenib or vemurafenib as single agents showed clinical benefit and safety (Brose et al. 2016; Falchook et al. 2015; Rothenberg et al. 2015), not inferior to combined therapy (dabrafenib + trametinib). In fact, combination therapy had a higher incidence of severe adverse events compared to dabrafenib alone (Busaidy et al. 2022).

ATC usually presents as unresectable tumors with distant metastasis. This diagnosis must be followed by a prompt search for *BRAF* V600E, as the combination therapy with dabrafenib and the MEK1/2 inhibitor

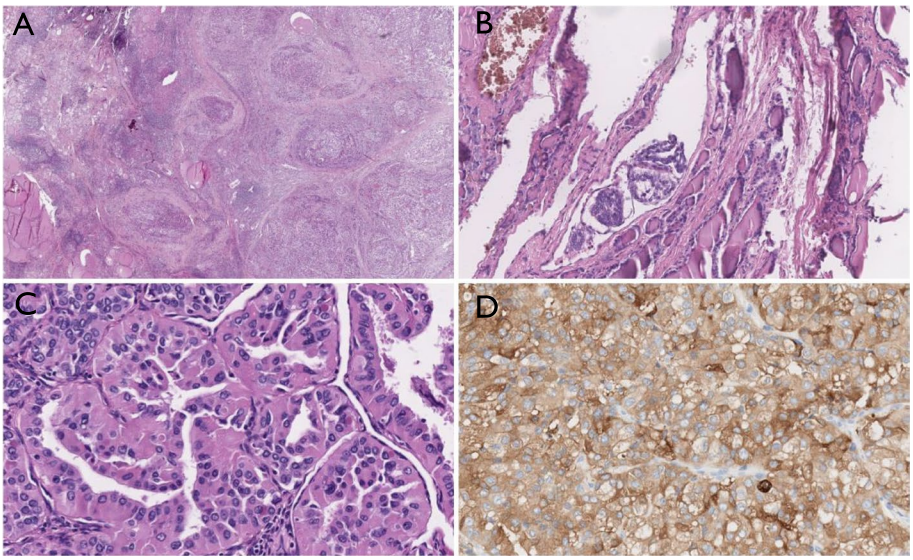

Fig. 4 Example of a PTC with *NTRK1* fusion. There is multinodular growth (**A**), oncocytic features (**C**) and frequent lymphatic invasion (**B**). Immunohistochemistry shows strong cytoplasmic staining on neoplastic cells (**D**). Images from the Archives of Anatomic Pathology Department, Rede D'OR São Luiz

trametinib is currently approved by the FDA for *BRAF* V600E-mutant ATC. In a phase II study with 16 patients, the ORR was 69% and the median duration of response (DoR), Progression-Free Survival (PFS), and Overall Survival (OS) in 12-months were 90%, 79%, and 80%, respectively (Subbiah et al. 2018).

NTRK (Neurotrophic Tropomyosin Receptor Kinase)

The family of TRK transmembrane receptors A, B and C, encoded by *NTRK* 1, 2 and 3 genes, are responsible for transduction of signals crucial for the development of neural cells and the maintenance of normal neuronal balance in adult tissues (Manea et al. 2022; Marchetti et al. 2022; O'Haire et al. 2023; Hechtman 2022). They are composed of an extracellular ligand-binding domain, a transmembrane domain and an intracellular kinase domain. Receptor's homodimerization after interaction with the ligand, followed by phosphorylation of cytoplasmic Kinase domains are necessary to initiation of downstream signaling pathways (Solomon et al. 2019). In *NTRK* fusions, the C-terminal Kinase domain of the *NTRK* gene join the N-terminal portion of the fusion partner, causing constitutive activation of the TRK pathway, with oncogenic potential in both neural and non-neural cells (Manea et al. 2022; Solomon et al. 2020). Only fusions, but not *NTRK* mutations, splice variations or amplifications (which have been explored in some types of solid tumors with uncertain oncogenic potential) are actionable and therefore have a role as predictive biomarker (Hechtman 2022).

NTRK fusions are seen in numerous malignant solid tumors of various histologic types, but they are also remarkably rare, detected in less than 1% of the cases. Conversely, they are quite common and the defining genetic aberration of some uncommon malignancies such as the secretory carcinoma of various sites and the infantile fibrosarcoma with *ETV6-NTRK3* fusion. Thyroid tumors have a relatively higher frequency of *NTRK* fusions than other solid tumors, with notably variable rates in different studies: from 1.68% to 6.7% of PTCs and 14.5% of irradiation-associated thyroid carcinomas have *NTRK* fusions and that frequency can be 8-fold higher in children. Of note, *NTRK* fusions are not restricted to PTC and can also be found in FTC, OC, PDTC, ATC and MTC (Manea et al. 2022; Marchetti et al. 2022; O'Haire et al. 2023; Hechtman 2022; Solomon et al. 2020; Pekova et al. 2021; Park et al. 2022; Waguespack et al. 2022).

Fusions described in thyroid carcinoma involve mostly *NTRK1* and *NTRK3*, the *ETV6-NTRK3* fusion being the most common, seen in 64.4% (Pekova et al. 2021) of the cases. Importantly, non-secretory thyroid carcinomas with *NTRK* fusions are generally more aggressive. They usually show as multinodular lesions composed of oncocytic cells arranged in a mixture of papillary, follicular or solid patterns; high grade features are often present, characterized by increased mitotic figures, extensive lymphovascular invasion and extrathyroidal extension (Fig. 4) (Chu et al. 2020). One study found that thyroid

carcinomas with *NTRK1* fusions have significantly more features of aggressiveness than the ones with *NTRK3* fusions, with more frequent lymphovascular invasion, extrathyroidal extension and lymph node and distant metastasis (Pekova et al. 2021).

In situ (IHC, FISH) and in vitro (Real Time-PCR, NGS) ancillary techniques can be applied to detect *NTRK* fusions, and their performances are dependent on the sample quality and the tumor type. IHC may show cytoplasmic or membrane staining in the presence of *NTRK1/2* and nuclear staining in *NTRK3* fusions. However, its sensitivity is dependent on pre-analytical factors, and it is generally lower for *NTRK3*-fusion+ carcinomas. RNA-based NGS has the best sensitivity (95.3%) among all techniques and a specificity of 100%; it has the advantage over DNA-based NGS due to the identification of the precise fusion partner, as well as the chimeric transcripts. DNA-based NGS has lower sensitivity, partly attributed to large intronic regions of *NTRK2* and *NTRK3* that are generally uncovered by the test. As a result of this obstacle, for *NTRK3* fusions, commercial probes often cover only its most frequent partner *ETV6*, meaning that other *NTRK3* fusions with other partners would not be detected. However, DNA-based NGS can be valuable in identifying amplifications, deletions and point mutations in many other genes as *RAS* or *BRAF*, which are mutually exclusive with *NTRK* in thyroid tumors; moreover, it can identify *NTRK* point mutations associated with resistance to TRK-inhibitors. Therefore, the application of DNA/RNA-based NGS panels can combine the advantage of both methods in this context (Hechtman 2022; Marchiò et al. 2019; Conde et al. 2021; Solomon and Hechtman 2019).

TRK-inhibitors have tumor-agnostic approval for treatment of tumors with *NTRK* fusions in adult and in patients over 12 years old. Larotrectinib is a first-generation, highly selective TRK inhibitor, demonstrating ORR of 78% and median DoR of 43.3 months for patients with DTC with distant metastasis in one study (Cabanillas et al. 2023). Entrectinib, another first-generation inhibitor, target also ALK and ROS1 tyrosine kinases, and despite the capacity of crossing the blood–brain barrier, it seems to be less effective for thyroid tumors than for other solid tumors with TRK-fusion. Few studies with the second-generation Taletrectinib also showed efficacy on treatment of advanced DTC (Cabanillas et al. 2019; Park et al. 2022; Cabanillas et al. 2023; Papadopoulos et al. 2020; Doebele et al. 2020).

RET (REarranged during Transfection)

RET encodes a Tyrosine Kinase (TK) transmembrane receptor essential to the development of the kidney, male germ cells and neural crest cells, and therefore is expressed on thyroid C cells, ganglia of the peripheral nervous system and in some parts of the central nervous system in normal adults. It is composed of a large extracellular domain, a transmembrane domain and an intracellular kinase domain like other TK receptors. Four ligands (GDN, NRTN, ARTN, PSPN) constitute a protein complex that induce RET activation after interaction between growth factors with specific coreceptors. Then, the process is followed by dimerization and phosphorylation of the kinase domain, the latter responsible for downstream signaling of multiple pathways, such as MAPK-ERK, PI3K-AKT and JAK-STAT, involved in cell growth, differentiation, survival and migration (Romei and Elisei 2012; Vodopivec and Hu 2022; Wagner et al. 2012; Santoro and Carlomagno 2013; Belli et al. 2021).

The first oncogenic alteration identified in PTC was a chimeric fusion involving *RET* and *CCD6* by the end of the 1980's, named years later as *RET*/PTC. In 1993, germline activating mutations of *RET* in association of MTC and MEN 2A were first published (Mulligan et al. 1993; Lemoine et al. 1989; Fusco et al. 1987). Since then, numerous other fusions and sporadic or germline mutations affecting *RET* have been described, along with groundbreaking targeted therapies, making *RET* a robust predictive biomarker in thyroid cancer.

There are at least 19 *RET* fusions reported in association with thyroid cancer. They are almost always related to PTC and PDTC, and more than 90% of them correspond to *RET*/PTC1 (*RET-CCDC6*) and *RET*/PTC3 (*RET-NCOAA4*) rearrangements. Exceptionally, *RET* fusions can be found in MTC, as in the case of *MYH13-RET* translocation (Vodopivec and Hu 2022; Belli et al. 2021; Grubbs et al. 2015).

A transcriptionally active fusion of *RET* gene involves its TK domain in the 3' sequence and the 5' sequence of the fusion partner, which takes transcriptional control over *RET* (constitutive activation). As a result, RET protein is abnormally expressed in cells in which it is not normally present and, additionally, dimerization sites from the partners lead to ligand-independent activation of downstream pathways by the fusion protein. The abnormal activity of RET causes chronically sustained growth and proliferation stimuli and is a major driver in the development not only of thyroid tumors, but tumors from multiple other sites (Romei and Elisei 2012; Santoro and Carlomagno 2013; Belli et al. 2021).

Five to 25% of PTCs have clonal *RET* fusions, and they are mutually exclusive with other drivers of thyroid carcinoma. The variability of this frequency is influenced by population characteristics, detection methods and the PTC subtype. As a matter of fact, there is a close relationship between *RET* fusions, particularly *RET*/PTC3 and

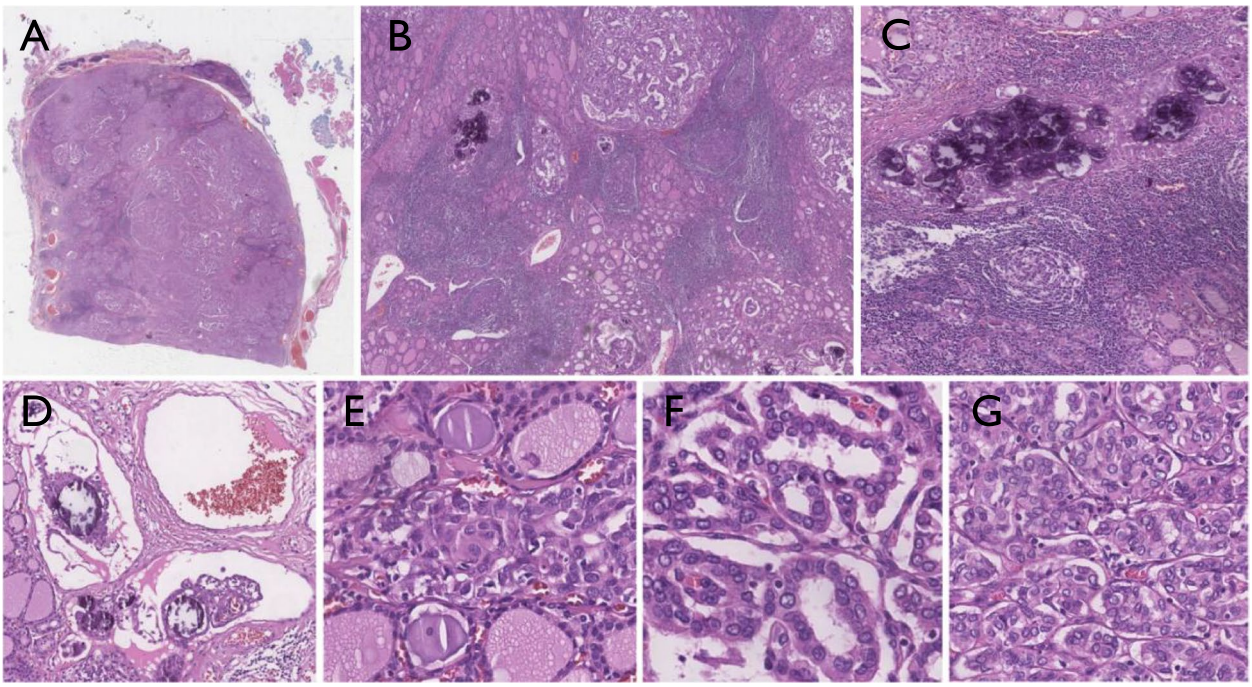

Fig. 5 *RET* fusions are the main molecular driver in the DSPTC. In this example, the tumor involved the entire gland (**A**) with associated Hashimoto's Thyroiditis (**B**), numerous psammoma bodies (**C**), extensive lymphatic invasion (**D**), squamous differentiation (**E**) and showed a mixture of follicular (**F**) and solid (**F**) patterns. Images from the Archives of Anatomic Pathology Department, Rede D'OR São Luiz

radiation exposure, including atomic bombs and nuclear accidents survivors, in which the translocation can be found in 50–80% of the cases. PTC in this background is typically of the solid/trabecular subtype and shows a short latency time from the exposure and a more aggressive behavior. *RET* fusions are also the dominant driver in the context of pediatric PTC and in the Diffuse Sclerosing Subtype of PTC (DSPTC), which is characterized by diffuse involvement of the thyroid, mixture of patterns, extensive lymphovascular invasion, numerous psammoma bodies and a background of Hashimoto's Thyroiditis, along with more propensity to nodal and pulmonary metastasis (Fig. 5 (Romei and Elisei 2012; WHO Classification of Tumours Editorial Board 2022; Belli et al. 2021; Gallant et al. 2022; Galuppini et al. 2019; Joung et al. 2016).

Somatic and germline mutations of *RET* play crucial roles in the development of sporadic and familial forms of MTC, respectively. Germline variants of the gene are present in more than 99% of patients with hereditary MTC, while somatic variants are present in 50–93.8% of patients (Elisei et al. 2008; Barletta et al. 2021b). The most common somatic *RET* variant is M918T, present in up to 40% of MTCs. It is associated with aggressiveness and response to targeted therapy. Other somatic *RET* variants at residues C611, C618, C620, C630, C634, E768, A883, and S891, as well as small RET deletions and/or insertions have been detected in MTC (Heilmann et al. 2016; Salvatore et al. 2021). Point mutations in *RAS* (*HRAS* and *KRAS*) represent another driver related to sporadic MTC, occurring in 20–50% of patients without *RET* mutations. Recently, inactivating variants of the *NF1* gene have also been found to be a driver of non-*RET*/non-*RAS* MTC (Ciampi et al. 2023; Castroneves et al. 2024). In approximately 20% of sporadic MTCs no variants in *RET* or *RAS* are identified, prompting ongoing studies to identify other drivers associated with MTC (WHO Classification of Tumours Editorial Board 2022; Agrawal et al. 2013).

RET alterations can be detected indirectly by IHC or by FISH, PCR and NGS platforms all of which can be executed on FFPE tissue, frozen samples or cytological specimens. PCR and NGS-based testing can also be performed on liquid biopsies. Since the abnormal expression of RET in tissue samples can be seen in benign thyroid nodules, the use of IHC is precluded, especially for therapeutic purposes. Given the number of possible partners, FISH break-apart probes are more appropriate than fusion probes, emphasizing that deletions and pericentric fusions can decrease their sensitivity. DNA-based NGS is another choice for detection of *RET* mutations and fusions with high sensitivity and specificity, but for fusions, breakpoints that occur in several long intronic sites must be covered. As a matter of fact,

the problem with the intronic regions can be solved by RNA-based NGS, as they are spliced during transcription. Other advantages of RNA-based NGS are the fact that only in-frame fusions are detected, along with better characterization of novel or atypical structural variants found in DNA-based NGS and the determination of the exact partner gene (Belli et al. 2021; Yang et al. 2021). All patients with diagnosed MTC should undergo genetic counseling and be tested for germline *RET* mutations (Shonka et al. 2022).

Carriers of germ line *RET* mutations typically undergo prophylactic or therapeutic thyroidectomy; the age of prophylactic thyroidectomy is determined by the mutation's aggressiveness. The 2015 American Thyroid Association (ATA) guidelines classify *RET* mutations into three categories: highest risk (M918T), high risk (codon 634 and A883F), and moderate risk (all other RET mutations). Prophylactic thyroidectomies are recommended before age 1 for highest-risk mutations, before age 5 for high-risk mutations, and within the first and second decade of life for moderate-risk mutations, depending on the mutation's aggressiveness and its behavior within the affected family (earliest age of onset, disease-free survival, and disease-specific mortality) (Haugen et al. 2016).

The identification of *RET* abnormalities has ushered in a new era of treatment options for patients with metastatic disease. Modern and highly specific RET inhibitors selpercatinib and pralsetinib were approved by the FDA in May and September of 2020, respectively, for treatment of *RET*-altered carcinomas. LIBRETTO-001 was a phase 1–2 clinical trial investigating safety and efficacy of selpercatinib in 162 patients with MTC and 19 patients with RAIR carcinomas with *RET*/PTC1 fusions (13 PTC, 3 PDTC, 2 ATC and 1 OC). In the *RET*-mutant MTC group, the ORR was 73% and the 1-year PFS was 92% in patients without previous treatment with vandetanib or cabozantinib. In the previously treated subgroup, the ORR was 69% and the 1-year progression-free survival was 82%. Only 2% of patients discontinued treatment because of adverse events. The ORR for the RET-fusion subgroup was 79% for all tumor types, with a 1-year PFS of 64% (Wirth et al. 2020).

The ARROW study, also a phase 1/2, open-label trial, included 84 patients with *RET*-mutant MTC (61 patients pre-treated with cabozantinib or vandetanib or both, and 23 treatment-naïve patients) and 32 patients with carcinomas harboring a RET fusion (10 PTC and 1 PDTC) that were treated with pralsetinib. The ORR was 71% in treatment-naïve patients and 60% in pre-treated patients in the MTC subgroup. The median PFS and OS were not reached, but the estimated 1-year PFS after a median follow-up of 15 months was 75% in pre-treated patients and 81% in treatment-naïve patients. In the DTC subgroup, the ORR was 89% and the 1-year PFS was 81%. Treatment was discontinued due to serious adverse effects in 4% of the participants (Subbiah et al. 2021).

TERT (Telomerase Reverse Transcriptase)

Telomeres are repeated sequences of DNA associated with proteins, found at the end of chromosomes, mostly responsible for their stability. In human cells, successive replication causes shortening of telomeres, which is associated with replicative senescence, limiting the number of divisions in the cell life spam and decreasing tissue functionality. TERT is the catalytic subunit of telomerase enzymatic complex, responsible for keeping the telomere's length and integrity and therefore leading to senescence arrest. It is normally silent in normal cells, but is frequently reactivated in human in cancer, when various telomere maintenance mechanisms can take place: *TERT* gene rearrangements, amplifications, somatic mutations, among others (Yuan et al. 2019; Victorelli and Passos 2017; Dratwa et al. 2020).

In thyroid cancer, the presence of *TERT* mutations, mainly represented by the most frequent C228T and the C250T variants, is associated with older age, large tumor size and aggressive behavior in DTC. Notably, they are more often detected in PDTC (43.2%) and ATC (40.1%) than in PTC (11.3%) and FTC (17.1%) (Liu and Xing 2016; Yang et al. 2022). The value of *TERT* mutations as a predictive biomarker in thyroid carcinomas relies on the fact that their presence is associated with a RAIR phenotype in metastatic DTC, with a synergistic negative effect with the co-occurrence of *BRAF* V600E (Mu et al. 2022; Yang et al. 2017).

Microsatellite Instability (MSI), Tumor Mutation Burden (TMB), PD1/PD-L1 (Programmed Cell Death Protein 1/ Programmed Cell Death Ligand 1) and immunotherapy in thyroid cancer

Microsatellites are repetitive, often non-coding, DNA sequences present throughout the genome along with other types of repetitive sequences implicated in evolution and gene expression regulation (Liao et al. 2023). Microsatellite Instability (MSI) refers to the variation on the length of these sequences due to insertions and deletions that can occur during cell division. When such defects could not be corrected by the Mismatch Repair (MMR) Proteins apparatus or other mechanisms, a MSI phenotype develop, allowing mutations to accumulate both in the genes harboring those microsatellites and other genes, resulting in an anticipated hypermutated state, a key event for development of malignant tumors. Mutations in the genes coding MMR proteins can happen sporadically or in hereditary fashion, the latter

prototypically represented by the Lynch Syndrome (Shia 2021; Yamamoto and Imai 2019; Büttner et al. 2019; Latham et al. 2018).

MSI can be found more often in association with certain tumor types, such as colorectal (CRC), endometrial (EC), small bowel, bladder and adrenocortical cancers (Latham et al. 2018). In thyroid, given the usual low total number of mutations (TMB) seen in most carcinomas (Lu et al. 2021), it may be expected that MSI will concordantly be a rare event. However, conflicting results have been published regarding the frequency of MSI in thyroid neoplasms, which can range from 0% to > 80% depending on the study, with FTC apparently excelling other tumor types (Genutis et al. 2019). These disparities most likely are due to technical or methodological issues. In fact, most PCR- and sequencing-based tests used to assess MSI were designed in the past to detected abnormalities in microsatellites mainly related to CRC and EC, which are canonical tumors of LS. Aware of that, some authors analyzed spots of microsatellites related to different genes, such as *RET, p53, TSHR* (Thyroid Stimulating Hormone Receptor gene) and *THRA1* (Thyroid Hormone Receptor alpha), all of them implicated in thyroid carcinomas, resulting in an exceptional high rate of MSI in sporadic DTC. In that study, not only MSI was detected in all the above-mentioned microsatellites, but in *D2S123*, which was altered in 100% of FTCs. Of note, *BAT-26*, a microsatellite classically described in CRC, had the lowest rate of MSI (Onda et al. 2001). Moreover, thyroid carcinomas can also occur as non-canonical manifestation of LS, when they are mostly associated with MSH2 abnormalities. In this setting, MSI may not be detected by molecular testing using conventional panels, despite the loss of expression of MMR by immunohistochemistry (Stulp et al. 2008; Broaddus et al. 2004).

Detection of MSI and TMB is preferably done by NGS-based platforms. There are several commercial tests suitable for FFPE samples, but it is important to remember that, as already mentioned, an MSI-High result may be not achieved for tumors that are non-canonical in the LS, even if they are unstable (Shia 2021). Therefore, immunohistochemical search for loss of expression of one or more MMR proteins is an important adjuvant in this investigation.

The greater the tumor TMB, the larger the amount of neoantigens in the tumor microenvironment will be, theoretically triggering a more intense T-cell mediated immune response by the host. Programmed cell death-1 (PD-1) and its ligands programmed cell death ligand-1 and 2 (PD-L1 and PD-L2) are chief immune checkpoint molecules involved in downregulation of T-cell-mediated inflammatory response in physiological and pathological conditions. PD1 can be expressed on activated T cells and other immune cells; PD-L1 is expressed on healthy tissues and on immune cells in response to various cytokines, whereas PD-L2 is mainly present on activated dendritic cells and macrophages. During tumor development, the immune response evoked by neoantigens in the tumor microenvironment can be suppressed by the interaction between PD-L1 expressed on tumor or immune cells with PD-1 on the immune cells. This mechanism of immune evasion by the tumor is one the hallmarks of cancer and is the biological basis for contemporary immunotherapy (Jiang et al. 2019; Seliger 2019; Hanahan 2022)

PD-L1 has been evolving as a diagnostic and prognostic biomarker in thyroid cancer: meta-analyses showed that PD-L1 is associated with poor survival in DTC, PDTC and ATC, but apparently not in MTC (Wan et al. 2021; Girolami et al. 2020; Aghajani et al. 2018; Wusiman et al. 2024). Overexpression of PD-L1 in DTC was related to tumor recurrence, associated thyroiditis, extrathyroidal extension, late stage of DTC, and *BRAF* V600E mutation (4,5,8,9). Furthermore, limited data suggests that PD-L1 expression can help to distinguish NIFTP from IFVPTC (Ulisse et al. 2019).

As for its predictive value, PD-L1 expression is still used as determinant biomarker for treatment with immune checkpoint inhibitors in multiple tumors, such as lung, stomach, urothelial, head and neck cancer, among others, but for some other cancers, like malignant melanoma, the use of such agents is independent on PD-L1 status. In practice, PD-L1 is assessed routinely at protein level by IHC, with expression scores for different commercial clones validated according to the tumor type, but it can also be comparably measured through the detection of mRNA by NGS (Charifa et al. 2023). Although PD-L1 can be expressed in about 53.2% of PTCs (Bai et al. 2017) and across other thyroid tumors, like PDCT, ATC and MTC, there are no expression scores validated so far (Wan et al. 2021; Girolami et al. 2020; Aghajani et al. 2018; Wusiman et al. 2024; Agarwal et al. 2021).

Immunotherapy was approved for thyroid carcinoma by the FDA in 2020 (pembrolizumab for cases with TMB-high (> 10mut/MB)) and can also be considered in MSI-high or dMMR tumors with no other satisfactory treatment options (Agosto Salgado et al. 2024). Nonetheless, modest response was observed in patients with ATC regardless of MSI or TMB status in one study (Khan et al. 2018). Another work found an ORR of 19% in patients with ATC treated with spartalizumab (8 of 42 patients, 3 patients with complete response); notably, the best responders had PD-L1 expression > 50% and 1-year survival was of 52.1% for that population (Capdevila et al. 2020). Even though immunotherapy with single agents have not replaced conventional strategies for patients

with advanced thyroid cancer, alternatives combining different checkpoint inhibitors or other types of immune blockade, as well as the association of immunotherapy with TKIs seem to be promising and need further development (French 2020).

Conclusion

For the small but significant proportion of patients with advanced, metastatic thyroid cancer in need of systemic treatment, the study of predictive biomarkers can select the cases who might benefit from the use of modern, highly specific TKIs. For this purpose, the recognition of the molecular-morphological correlation between main genetic drivers and different types of thyroid carcinoma at the initial morphological diagnosis, along with the availability of proper technological apparatus supported by best pathology laboratory practices and multidisciplinary work are vital to ensure the highest accuracy and the shortest turn-around time. Additionally, studies on novel biomarkers and drugs for the patients with thyroid cancer that remain without a targetable alteration are needed.

Authors' contributions

H.C.C performed review concept and design, image acquisition and design, writing, and final revision; R.A.N performed writing and table edition; E.C.F performed *RET* structure and writing; A.A.B performed *BRAF* structure and writing; L.G performed *MSI/TMB* structure and writing; A.M.P.S.B performed writing; A.A.F.O.H performed coordination, writing and editing. All authors read and approved the final paper.

Funding

The authors received no specific funding for this work.

Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors have no conflicts of interest to declare.

Received: 30 June 2024 Accepted: 15 July 2024
Published online: 14 August 2024

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