Molecular testing for thyroid nodules: Where are we now?

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Abstract

Approximately 25% of the fine needle aspiration samples (FNAB) of thyroid nodules are classified as "indeterminate samples", that means, Bethesda III and IV categories. Until the last decade, most of these cases underwent diagnostic surgery, although only a minority (13–34%) confirmed malignancy postoperatively. In view of this, with the objective of improving the preoperative diagnosis in these cases, the molecular tests emerged, which are validated from the diagnostic point of view, presenting good performance, with good diagnostic accuracy, being able to avoid diagnostic surgeries. With the advancement of knowledge of the role of each of the mutations and gene rearrangements in thyroid oncogenesis, molecular markers have left to play only a diagnostic role and have been gaining more and more space both in defining the prognostic role of the tumor, as well as in the indication of target therapy. Thus, the objective of this review is to show how to use the tool of molecular tests, now commercially available in the world, in the management of indeterminate cytological nodules, assessing the pre-test malignancy risk of the nodule, through clinical, ultrasonographic and cytological characteristics, and decide on the benefit of molecular testing for each patient. In addition, to discuss its new and promising prognostic and therapeutic role in thyroid cancer.

Keywords Thyroid nodule · Indeterminate cytology · Molecular tests · Molecular markers

1 Introduction

Thyroid nodules are very common, and their clinical impact consists of excluding malignancy, local symptoms of compression and autonomous hormone production of the tumor [1].

When the objective is to exclude malignancy, although ultrasound-guided fine needle aspiration (FNAB) is still the gold standard method [2], approximately 25% of the samples do not have the necessary information for a definitive diagnosis and are classified as "indeterminate samples", that is, classes III (atypia of undetermined significance - AUS) and IV (follicular neoplasm) in the Bethesda classification [3]. Although Bethesda V is also classified as "indeterminate" (suspicious for malignancy), the risk of malignancy is significantly higher (67–83%) [3] when compared to the other two, and thus the management of Bethesda V nodules differ from Bethesda III and IV, approaching a more similar treatment for Bethesda VI (malignant).

Until the last decade, most nodules with Bethesda III and IV cytologies underwent diagnostic surgery. Due to the fact that only a minority of the cases (13–34%) presented malignancy postoperatively [3], it was necessary to improve the preoperative diagnosis of these samples, in order to reduce unnecessary surgeries and the consequent expenses and risks. For this purpose, in recent decades, several groups have dedicated themselves by characterizing the molecular profile of thyroid nodules, especially differentiated thyroid carcinomas (DTC). This information was brought to clinical practice and different molecular tests were developed and are being commercialized nowadays.

Molecular tests already have their indication recommended by the main guidelines for the treatment of thyroid nodules and are already progressing to individualize treatments, based on the genetic information found. In addition, the genetic information obtained can help to guide targeted therapies. Next, we will see when we can use this diagnostic tool and how to use molecular information prognostically to guide a more individualized treatment and how to use this information in the indication of targeted therapies. To

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facilitate the understanding of the indication for diagnostic and prognostic molecular testing, we will consider indeterminate nodules in Bethesda categories III and IV.

2 Management of nodules with indeterminate cytology

Indeterminate nodules have a very variable risk of malignancy (ROM), that means, from 13 to 34% [3]. Due to this wide range, it's important to make use of several diagnostic methods with the aim of refining the ROM and indicating surgery with greater accuracy for those lesions with a higher chance of malignancy. Thus, towards an indeterminate sample, we will use the information from the ultrasound (USG) - risk of malignancy, size, location, and nodule growth; patient history (gender, age, family history of thyroid cancer, personal history of radiation exposure); classification and description of greater risk on cytology (if category III with nuclear atypia and category IV), review or repeat of FNAB and molecular tests.

In a practical way, according to the current published information, it seems that the "graduated multimodal approach" [4] is a feasible method to help us in clinical practice to manage Bethesda class III and IV samples (Fig. 1).

Faced with an AUS sample (Bethesda III), the recommended management is to repeat FNAB [3]. Some studies have shown that a new FNAB can redefine the diagnosis in 75% to 80% of cases [5]. In cases of Bethesda IV samples, repeating the FNAB does not seem to be beneficial [3], if it was performed in a reliable service.

Since core biopsy has not yet confirmed clear advantages in relation to its cost–benefit, its indication must be individualized. In some cases of Bethesda, I class or by repeating a biopsy in a Bethesda III class sample, it may be considered, if available [6].

If the cytology remains indeterminate after a new FNAB/core biopsy or a slide review, it is affordable to take a step back and check again the following elements in patient's history: (a) personal head and neck radiation exposure, familial history of DTC (3 or more first-degree affected members) [7], medullar thyroid cancer or multiple endocrine neoplasia type 2, (b) characteristics of the nodule: nodule location (near vital structures as trachea or in the path of the recurrent laryngeal nerve), size (> 4 cm or compressive symptoms) [2]; sonographic characteristic: ACR-TIRADS or ATA risk stratification; (c) cytological description: presence of architectural and/or nuclear atypia; and (d) calcitonin levels. Thus, it is possible to determine, through these findings, which are those with the lowest ROM and those with the highest ROM, in order to define the nodules that can be followed up clinically, which ones would benefit from a molecular test, or which ones should be treated with surgery (Fig. 1).

In cases of TIRADS 3 and 4, without significant familial or personal history, molecular testing may be indicated [3].
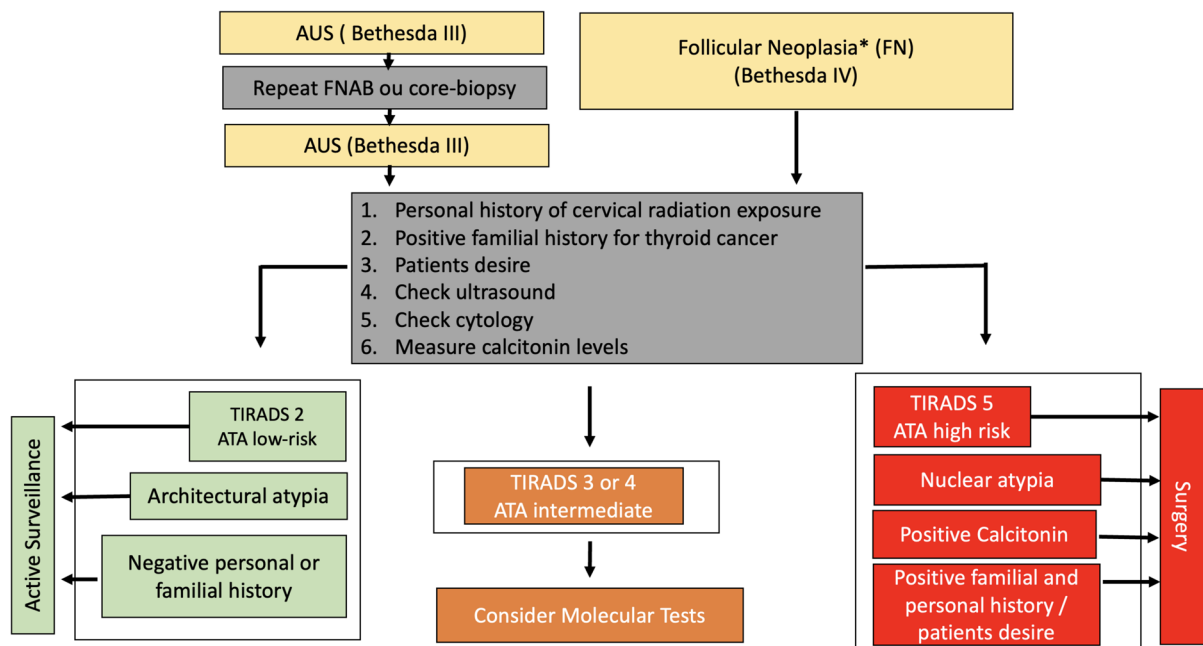

Fig. 1 Practical scheme to guide the management of thyroid nodules with indeterminate cytology (*In case of Bethesda IV cytology, there are not always benefits in repeating the FNAB. A new FNAB can be avoided if it has already been performed in a reliable service or evaluated by an experienced thyroid cytopathologist)

In these cases, to opt for one of the molecular tests, test performance, regional cost, availability and which genetic information doctors want to obtain (whether diagnostic x prognostic x therapeutic information).

In the case of a patient with no history of radiation exposure or familial cancer, with low-risk ultrasound image and only architectural atypia, as the ROM is low, thus, active surveillance seems to be the best initial approach for the management of this nodule. Important to say is that although epidemiologically we know that DTC is more frequent in women than in men, a worse prognosis in men is still in debate and poorly explained [8]. There are some theories that could explain it, like the role played by estrogen during the fertile period duration, limiting and minimizing oncogenic processes [8] but currently, no guideline recommends a more aggressive approach in men as an independent risk factor compared to women.

On the other hand, a patient that has positive personal or familial history, high risk ultrasound images, nuclear cytological atypia, high calcitonin levels (> 30 pg/mL for female and 34 pg/mL for male) [6] or nodules > 4cm [9] immediate surgery seems to be the most appropriate management. According to age, patient < 18 years old should be treated with surgery, since there are insufficient available data of validation of molecular tests for this group [10].

3 Molecular tests

The molecular tests commercially available basically use two types of technologies, thus categorizing the molecular information into (1) classifiers and (2) markers. The "classifier" tests use the genetic information of genes or microRNAs (miRNA), whose expression levels can define a profile of benignity or malignancy of a nodule. This type of marker usually presents negative predictive values (NPV) and high sensitivity, characterizing a good profile to exclude the presence of malignancy, therefore called "rule-out". The classifiers, by this profile, have an important diagnostic power, thus well differentiating benign from malignant nodules in indeterminate samples of FNAB. The "marker" tests, on the other hand, use the presence or absence of known CDT mutations as genetic information. Because they have a high positive predictive value (PPV) and high specificity, this information is used as "rule-in" tests, that is, capable of confirming the malignancy of the analyzed sample [11]. Depending on the type of mutation present in the nodule, we can also confer prognostic power to the "marker" tests. Therefore, it can be said that, depending on the genetic information used, molecular markers play a role in the diagnostic definition or in the prognostic prediction, with an impact on defining the type of surgery and/or guiding therapeutic targets.

In addition, to divide a test into "rule-in" or "rule-out" we also need to have a very clear concept: the risk of pretest malignancy (ROM). According to Bayes' theorem, once the sensitivity and specificity of a test are fixed, whenever the prevalence of thyroid cancer changes, the PPV and NPV will be modified. Therefore, in an undetermined sample with a higher prevalence of cancer, the PPV will also increase and, consequently, there will be a reduction in the NPV. Likewise, a lower prevalence of cancer would increase the NPV and decrease the PPV. Therefore, when interpreting the performance of a test in a given population, knowledge of the ROM is of paramount importance in order not to draw hasty conclusions about the quality of a test, especially when compared to another.

To be considered a good rule-in test, it must have a PPV close to or > 70% and a specificity close to or > 87%. A good "rule-out" test should present a VPN close to or > 95% and sensitivity close to or > 86% [12]. These numbers are based on information from the Bethesda classification. That is, a desirable "rule-out" test would have the Bethesda class II performance to diagnose benign lesions, with a false-negative rate < 5% (NPV > 95%). Likewise, we want to identify malignant nodules as well as Bethesda classes V and VI, therefore implying a PPV > 70%. To get to these numbers, according to Bayes' theorem, we considered the ROM of 25%, which is the average malignancy found in Bethesda III and IV classes [10].

Using both, classifiers and markers information, in worldwide clinical practice, today, we have five commercially available tests for FNABs with indeterminate cytology: the new Afirma Genomic Sequencing Classifier (GSC; Veracyte, Inc., South San Francisco, CA, USA), Mir-THYpe (ONKOS Diagnostics Moleculars LTDA, Ribeirão Preto, Brazil) and ThyroPrint (GeneproDX, Santiago, Chile) which are tests with a "classifier" profile. The new version of ThyroSeqv3 (CBLPath, Inc, Rye Brook, NY, and University of Pittsburgh Medical Center, Pittsburgh, PA, USA), and ThyGenX/ThyraMIR (Interpace Diagnostics, LLC, Parsippany, NJ, USA) that have a profile "marker". Most of these tests are already in their second or third versions. With the evolution, the new versions of each test and the high NPV, PPV, sensitivity and specificity described, we can say that basically there is no longer an exclusively "rule-in" or "rule-out" test. With the inclusion of information from markers and classifiers, they perform both diagnostic and prognostic functions (Table 1).

4 Diagnostic role of molecular markers in DTC

The diagnostic role of molecular markers aims to differentiate benign from malignant nodules through the molecular information of samples with indeterminate cytology (Bethesda III and IV). For this purpose, we use tests known

Table 1 Summary of the current commercially available molecular tests for indeterminate samples

	Afirma GSC	Mir-THYpe	ThyGeNEXT/ThyraMIR	ThyroPrint	ThyroSeq
Molecular Material analyzed	Gene expression Mutation hotspots Fusions	miRNA expression	Mutations, Fusions, miRNA expression	Gene expression	Mutations Fusions CNA Gene expression
Technology	NGS - RNA Seq	qPCR	NGS / qPCR	qPCR	NGS
Sensitivity	91	95	89	91	94
NPV	96	96	94	95	97
Specificity	68	81	85	88	82
PPV	47	76	74	78	66
Validation	Yes	Yes	Yes	Yes	Yes
Studies	Patel et al. [13]	Santos et al. [16] Santos et al. [17]	Labourier et al. [18] Lupo et al. [19]	Gonzales et al. [20] Zafereo et al. [21]	Nikiforova et al. [22]
	Independent: Angell et al. [14] San Martin et al. [15]	Independent: No	Independent: No	Independent: No	Independent: Desai et al. [23] Jug et al. [24]

GSC genomic sequencing classifier, *qPCR* quantitative polymerase chain reaction, *NGS* next generation sequencing

as "rule-out" tests, meaning that, as described above, panels with high NPV and high sensitivity are employed. Thus, malignancy can be ruled out, and patients can be clinically monitored without the need for surgery, reducing morbidity and the cost of repeated FNABs.

The first molecular tests commercialized for this purpose emerged through the analysis of differentially expressed genes in benign and malignant samples, thus defining gene expression patterns of different tumor types called "classifiers." With the role of bioinformatics and the use of artificial models, computational algorithms were created, and these molecular classifiers were able to differentiate benign from malignant samples in indeterminate cases. This led to the development of the "Afirma Gene Expression Classifier" (GEC) by Veracyte, Inc. (South San Francisco, CA, USA), which is a classifier consisting of 167 genes aimed at identifying benign nodules with indeterminate cytology, initially described with high sensitivity and high NPV [25]. However, in some prospective and independent validations, it was observed that both the NPV and sensitivity fell significantly below the initially described values [26].

With the improvement of the algorithm and the analysis of a specific gene profile for Hürthle cells, the first version was replaced by the "Afirma Genomic Sequencing Classifier" (GSC) [13]. The new version, available since 2018, evaluates the expression profile of 1115 genes using RNA-Seq methodology. According to the data from initial validations, the GSC was tested on the same cohort used for the first generation of Afirma® GEC, showing an increase in specificity (from 53 to 68%) and PPV (from 38 to 47%), while maintaining high sensitivity and NPV from the original study. Furthermore, the GSC demonstrated higher specificity and PPV for Hürthle cell adenomas compared to the

GEC. Independent studies comparing the performance of GSC with GEC have been confirming these results [14, 15].

Using also a gene expression classifier, ThyroPrint, a 10-gene classifier, showed a NPV of 98%, PPV of 78%, sensitivity of 93% and a specificity of 81% at the first version [20]. The test is still in its first version and a second validation study in two different continents demonstrated equivalent performance, with a NPV of 94 and 96%, PPV: 74 and 82% and specificity of 89 and 87% in both continents (South America and North America, respectively) [21].

Another possible molecular diagnostic approach is through miRNAs and the identification of their expression profile in benign and malignant nodules. miRNAs represent a class of endogenous RNAs approximately 22 nucleotides long that act as post-transcriptional silencers, inhibiting the translation of messenger RNAs [27]. Therefore, the main function of miRNAs seems to be in gene regulation. Several studies have demonstrated the association of these small RNAs with the regulation of proliferation, apoptosis, differentiation, hematopoiesis, and they can function as oncogenes or tumor suppressor genes, thus playing a role in cancer diagnosis [28]. The expression of miRNAs in thyroid nodules has been demonstrated by various research groups. Three tests using miRNA expression have already been commercialized: ThyraMIR, Mir-THYpe, and RosettaGX, with the latter no longer being commercially available [11].

The Mir-THYpe® test is a Brazilian test developed and validated by the startup ONKOS Molecular Diagnostics. In this test, the genetic material (miRNAs) is extracted from the slides of the FNAB.

The test measures the expression of 11 miRNAs (6 normalizers and 5 discriminators) and identifies a pattern in the expression of these molecules. Using an artificial

intelligence algorithm, the expression pattern of the miR-NAs in the sample is compared to the expression pattern of other samples known to be benign or malignant, and based on this, the test generates a test result suggesting a "positive" or "negative" sample for malignancy. In validation, Mir-THYpe was able to correctly classify 82 out of 95 samples, with a sensitivity of 94.6%, specificity of 81%, NPV of 95.9%, PPV of 76.1%, and a diagnostic odds ratio (DOR) of 38.9%. However, it is worth noting that Bethesda V samples were included in the analysis, which may lead to an overestimated PPV [14]. Therefore, based on the published initial data, the Mir-THYpe test demonstrates high sensitivity and specificity, capable of accurately differentiating benign from malignant nodules in indeterminate samples [16].

Based on the information presented, the purpose of using a molecular test with a diagnostic role is to exclude malignancy in samples with indeterminate cytology, allowing patients to be clinically monitored and avoiding a diagnostic surgery. If a patient already has an indication for thyroidectomy, such as a nodule with a high suspicion of malignancy on ultrasound or compressive symptoms, there is no need to recommend a test with a diagnostic role.

Most tests have their diagnostic role well-established, and practically all current versions of the tests demonstrate high sensitivities and specificities in differentiating benign from malignant samples [11].

5 The prognostic role of molecular markers in DTC

Another important role of molecular tests is to provide prognostic information in DTC. They can assist in predicting tumor aggressiveness (risk of recurrence, extrathyroidal extension/invasion, local and distant metastases) and, consequently, in determining the extent of surgery (partial or total thyroidectomy, lymph node dissection) and the need for adjuvant radioiodine therapy.

There are several molecular tests that fulfill this role, such as Thyroseq v3, Xpression Atlas from Afirma, and ThyGenNext/ThyraMir. In addition to panels, there is also the possibility of analyzing individual mutations to aid in prognostic clarification. With this intention, we will now discuss the "rule-in" role of these tests, as well as detail the prognostic significance of the main mutations found in these panels.

It is known that in most cases of thyroid cancer, mutations are mutually exclusive events, meaning that only one of these mutations is found in any particular cancer [29]. When these mutations are used as independent biomarkers, their sensitivity and specificity are too low to be clinically relevant. However, the combination of analyzing these mutations in a panel has been shown to improve sensitivity and specificity rates [30]. Nikiforov et al. were the first group to

report sensitivity improvement (from 44 to 80%) and accuracy improvement (from 93.3% to 97.4%) by analyzing a panel of BRAF, RAS, RET/PTC, and PAX8/PPARγ mutation [31]. Based on this evidence, the "7-gene panel" was created using RT-PCR technique. This panel was the first prognostic test to be commercialized, and it showed high specificity values [31].

Next-Generation Sequencing (NGS) technology is a DNA sequencing technology that has revolutionized genomic research and emerged as an advancement over the Sanger sequencing method. Through this technique, multiple samples and multiple mutations can be analyzed simultaneously. Furthermore, with the publication of the integrated genomic characterization of PTC by The Cancer Genome Atlas (TCGA) [32], practically the entire genetic origin of PTC has been discovered, reducing the fraction of PTC cases with unknown oncogenic drivers from 25% to 3.5%. This offers a high potential for molecular diagnosis and opens the field to understanding the prognostic role of all these mutations.

Therefore, Thyroseq is a molecular test that uses NGS technology and has the ability to analyze a broad panel of mutations, rearrangements, and gene expression. Today, this test is already in its 3rd version (ThyroSeq v3) and evaluates oncogenic mutations in 112 genes (12,135 variants), over 120 gene fusions, copy number alterations in 10 chromosomal regions, and expression analysis in 19 genes [22]. In this panel, the authors created a "Genomic Classifiers Score" (GC score), where each genetic alteration and its levels (allelic frequency) detected were assigned a value from 0 to 2 based on the strength of their association with malignancy. The GC score ranged from 0–8, with < 1.5 being probably benign and > 1.5 being probably malignant. However, despite the high sensitivity and specificity found in this test in the original study as well as in validation studies, many of the mutations described still seem to lack correlation with their clinical functionality.

Another test that analyzes genetic mutations is ThyGeN-EXT, which can be used in combination with ThyraMir. ThyGeNEXT/ThyraMir together analyze the combination of the expression of 10 miRNAs and genetic mutations (10 in DNA and 37 in mRNA in 6 genes), with a sensitivity of 89%, negative predictive value (NPV) of 94%, specificity of 85%, and PPV of 74% in indeterminate samples [18]. Additionally, with the addition of TERT and ALK mutations, this panel has become capable of predicting the biological characteristics of tumor aggressiveness, thus also playing a prognostic role. However, there are no independent validation studies to corroborate these initial findings.

The Afirma Xpression Atlas (XA), marketed together with Afirma GSC (described earlier), had its latest version updated in 2020 and analyzes oncogenic mutations in 593 genes, including 905 variants and 235 fusion pairs. Recent data suggest that XA may have a prognostic role by

providing information about histology (distinguishing parathyroid, medullary carcinoma, CPT, etc.), tumor behavior, recognition of potential hereditary syndromes such as mutations in RET (Multiple Endocrine Neoplasia type 2), PTEN (Hamartoma Tumor Syndrome; Cowden Syndrome), APC (Familial Adenomatous Polyposis), and DICER1 (DICER1 Syndrome), and also have a potential role in suggesting the effectiveness of targeted therapy options (targeted agents) based on the mutations found [33].

The Mir-THYpe recently added the analysis of TERT and BRAF mutations, as well as the expression profile of miR-146b, to its miRNA expression panel, thereby also providing a prognostic role.

Although these molecular tests include the analysis of multiple genes to improve diagnostic power, from a prognostic standpoint, we need to understand the isolated role of each mutation in order to determine the appropriate course of action regarding the type of surgery (total or partial), the indication for radioiodine therapy, or targeted therapy with specific drugs. To better understand the individual prognostic role of the key mutations in thyroid cancer, we will now describe the most studied mutations to date.

6 BRAF V600E

The point mutation in codon 600 of the BRAF gene, resulting in the substitution of the amino acid valine with glutamate (V600E), was first described in melanoma and later in various types of cancer, including thyroid cancer. This mutation leads to constitutive activation of the BRAF kinase and MAPK signaling pathway (Raf-MEK-ERK), and it is the most common molecular alteration in papillary thyroid carcinoma (PTC) with a prevalence ranging from 36 to 69%. However, its prevalence is lower in the pediatric population, ranging from 3 to 48%.

Although the most studied mutation is BRAF V600E, its prognostic role, associated with increased tumor aggressiveness and poor prognosis in papillary thyroid carcinoma (PTC), is still subject to debate [34]. The BRAF V600E mutation is present in approximately half of classic PTC cases and in 40% of papillary microcarcinomas of the thyroid (mPTC), which generally have an excellent prognosis. In fact, the majority of individuals with BRAF-mutated thyroid cancer have favorable outcomes, resulting in a very low PPV of this mutation for worse outcomes per se [35]. Recurrence is the most commonly associated clinical and pathological feature with this mutation [35]. Therefore, this isolated mutation is a sensitive but not specific marker for recurrence and mortality in PTC. Based on these findings, it appears that the isolated BRAF mutation is not sufficient to contribute to stratifying PTC patients as high risk [2] and probably other pathways are likely to co-operate to promote cancer progression [36].

However, in the latest revision of the American Thyroid Association (ATA) guidelines, the presence of the BRAF V600E mutation is used to assist in risk stratification. Therefore, patients with papillary thyroid carcinoma (PTC) < 4 cm, N0M0, and wild-type BRAF have a 5-year recurrence risk of 1%, which increases to 8% when BRAF is mutated. Similarly, the impact of the BRAF mutation in multifocal non-incidental papillary microcarcinoma (mPTC) with extrathyroidal extension increases the recurrence risk to 20%, upgrading its risk category from low to intermediate [2].

Therefore, based on extensive research on this mutation, its isolated presence has an approximate specificity of 100% for papillary thyroid carcinoma (PTC) (diagnostic role), but alone, it does not seem to have a robust predictive role. However, according to the latest guideline, when present, the mutation has the power to reclassify some low-risk cases to intermediate risk of recurrence [2], and thus the current appropriate management for isolated BRAF V600E mutated tumor, in exception of mPTC, should be total thyroidectomy [2].

7 RET/PTC rearrangement

Rearrangements involving the RET proto-oncogene are commonly found in papillary thyroid carcinoma (PTC) (3–60%). RET/PTC1 and RET/PTC3 are the most common forms, representing over 90% of all rearrangements. They have also been described in pediatric PTC, both in sporadic form and in those exposed to radiation (15–76%) [37]. RET/PTC1 is more frequent in classic sporadic PTC that occurs in young patients and has been associated with non-invasive disease. On the other hand, RET/PTC3 [38] is more prevalent in the solid variant of PTC and seems to have a more aggressive behavior regarding size, multifocality, extrathyroidal extension, and solid-follicular growth pattern when exposed to radiation. This rearrangement has been frequently found in PTC in children affected by the Chernobyl accident [39].

The prognostic significance of RET/PTC is not yet fully established, but tumors harboring these alterations rarely progress to poorly differentiated carcinomas (PDTC) or anaplastic thyroid carcinomas (ATC) [40]. Therefore, in the presence of one of these mutations, the diagnosis of a PDTC is highly likely, with its prognostic implication still not well established.

8 Rearrangement PAX8/PPARG

The PAX8/PPARG rearrangement is most frequently observed in lesions with a follicular growth pattern (CFT and VFCFT) [32, 41]. It is also detected in benign lesions (14% of follicular thyroid adenomas - FTA). In some studies, the rearrangement has been associated with multifocality and vascular invasion, but there is not enough evidence to

define it as a prognostic indicator in DTC [40]. In the TCGA study, it was described in 4/484 (0.8%) of CPT, primarily in FVPTC [41].

Therefore, in clinical practice, the presence of the PAX8/PPARG rearrangement may not help us define malignancy diagnosis (as it is found in a significant percentage of benign lesions) or prognosis in tumors already diagnosed.

9 RAS

The oncogenic RAS family regulates two important signaling pathways in thyroid cancer: the MAPK cascade (Ras-Raf-MEK-ERK) and the PI3K/AKT pathway [42]. All three RAS genes have been found mutated in thyroid cancers, with NRAS codon 61 mutation being the most common, followed by HRAS mutation. RAS mutations occur in both benign and malignant thyroid tumors, including follicular adenomas FTA, follicular variant of papillary thyroid carcinoma (FVPTC), classical PTC and with variable frequency in poorly differentiated thyroid carcinoma (PDTC) and ATC. They are more frequent in FVPTC (30–45%), and when present in CPT, they are more prevalent in FVPTC (30–45%) [32, 42].

Due to the small size and short follow-up period of most series, it is not possible to confidently state that RAS mutations alone have prognostic value, although some authors have shown an increased frequency of distant metastases in patients with RAS mutation [43].

However, this likely reflects the coexistence of additional oncogenic alterations [43], reinforcing the concept that isolated RAS mutation in thyroid cancer is associated with limited tumor aggressiveness and a good prognosis [32, 42].

Therefore, this isolated mutation has low diagnostic sensitivity and specificity, and is insufficient for risk stratification in follicular-pattern thyroid tumors because it is widely detected across the entire spectrum of tumors with this pattern, ranging from benign lesions to high-risk malignancies [42].

10 TERT

The TERT promoter gene mutation was initially described in 2013 and is associated with certain types of malignant tumors (melanoma, gliomas, thyroid) and confers an increase in TERT gene promoter activity [44]. Activation of the MAPK and/or PI3K/AKT pathways by BRAF/RAS mutations leads to increased expression of ETS family transcription factors. BRAF and RAS can bind to this consensus binding site, leading to up-regulation of TERT expression [44, 45].

In different series, the prevalence of TERT promoter mutation has ranged from 7 to 50%, being present in 7%

to 22% of well-differentiated classical PTC, and with a higher prevalence in patients with PDTC or ATC– 30–50% [32, 44, 45].

Among thyroid cancers derived from follicular cells, TERT promoter gene mutation has been shown to be associated with aggressive characteristics, particularly when combined with BRAF or BRAF/RAS mutations [46]. These associations are related to worse outcomes, higher mortality, and distant metastases [46, 47].

Currently, there is sufficient evidence to suggest that this mutation, either alone or in combination with others, is a predictor of worse prognosis in differentiated thyroid cancer (DTC) [32, 46, 47]. Therefore, the presence of this mutation in a panel allows us to consider a tumor with greater aggressiveness and a poorer prognosis, which may require more aggressive treatment. However, there are still no precise guidelines regarding the management of this mutation, such as the specific type of prophylactic cervical lymph node dissection.

11 Other less frequent mutations

Among the less frequent mutations that have been well studied are TP53, PIK3CA and NTRK rearrangements.

These three mutations are usually late events and are present in more aggressive and undifferentiated PTC [32, 48, 49]. Therefore, the presence of these mutations in a panel should lead us to consider a tumor with greater aggressiveness and a potential worse outcome, for which more aggressive treatment may be indicated (total thyroidectomy, lymph node dissection, and radioiodine therapy). NTRK rearrangements are more prevalent in radiation-induced thyroid cancers [32], with lower prevalence in sporadic PTC. They also occur in the pediatric population [39]. Currently, they are used as therapeutic targets in the treatment of PTC.

12 miR-146-5b

miRNAs, as previously described, are small RNAs capable of suppressing the translation of target mRNAs through degradation or inhibition, thereby regulating the expression of many oncogenes or tumor suppressor genes [27]. Previous studies have reported that some miRNAs (such as miR-21, miR-146b, miR-221, miR-222, and miR-181b) are dysregulated in CPT compared to healthy patients [32, 50]. In particular, miR-146b-5p has been shown in studies to confer more aggressive characteristics in DTC, such as increased recurrence, larger tumor size, lymph node metastasis, and lower disease-free survival [50]. However, more data are still needed to suggest a more precise cut-off to better define the prognostic role of miR-146b-5p.

As stated, despite the better-defined prognostic role of the most prevalent mutations, the majority of mutations described in DTC and present in the most commercially available tests worldwide lack validation and even practical application in clinical practice. Figure 2 can guide us, based on current knowledge, to make some therapeutic decisions. However, when facing a less frequent mutation, the role of the physician is to interpret the genetic alteration within the complete clinical context, taking into consideration the patient's history (family history, exposure to radiation, age, comorbidities), ultrasound and cytology characteristics.

13 The therapeutic role of molecular testing

Currently, there are several drugs being studied for the management of iodine-refractory differentiated thyroid carcinomas (RR-DTC), including tyrosine kinase inhibitors, targeted agents, immune checkpoint inhibitors, and redifferentiation therapy. As the name implies, each of these drugs has a different mechanism of action, targeting different pathways and yielding different results and side effects. In this review, we will focus, in a summarized manner, on which mutations already have FDA-approved targeted therapies and the best means of identifying them.

With the advent of targeted therapies for the treatment of RR-DTC in the last decade, molecular markers have gained another important role: identifying mutations and guiding therapies. Selective kinase inhibitors provide a more personalized therapeutic approach with fewer side effects and a better therapeutic response [51].

Among the commercially available molecular panels for identifying these mutations, the most specific ones for thyroid cancer are ThyroSeqV3, Atlas Afirma, ThyGenExt, and mirTHYpe Target. Additionally, other non-directed NGS panels, such as Foundation One, are also used in practice depending on the availability of each oncology service.

An important question that arises is which material to analyze: should we look for the driver mutation in the primary tumor in the thyroid, or should the material come from the metastases we intend to treat? There is still no consensus on this matter, and even in targeted drug trials, various different materials have been analyzed. However, in practice, we have been using the most recent and freshest available material. Another important point to consider when requesting a molecular test is which genetic material (DNA? RNA?) will be analyze.

When using RNA (such as Afirma), there may be a lower detection of point mutations like BRAF and RET, for example. Similarly, analyzing only DNA limits the detection of gene fusions [52]. The best material to be used in practice today is paraffin-embedded tissue blocks.

Currently, the mutations that already have targeted therapies are BRAF mutations, RET mutations, and NTRK fusions (Fig. 3).

14 BRAF inhibitors

BRAF mutation, as described, is the most frequent mutation in thyroid cancer. As a result, it has become a highly attractive therapeutic target. Dabrafenib, in combination with Trametinib (an MEK inhibitor), was approved by the FDA in 2018 for the treatment of anaplastic thyroid carcinomas [53] and has been approved since 2022 for the treatment of progressing cancer.
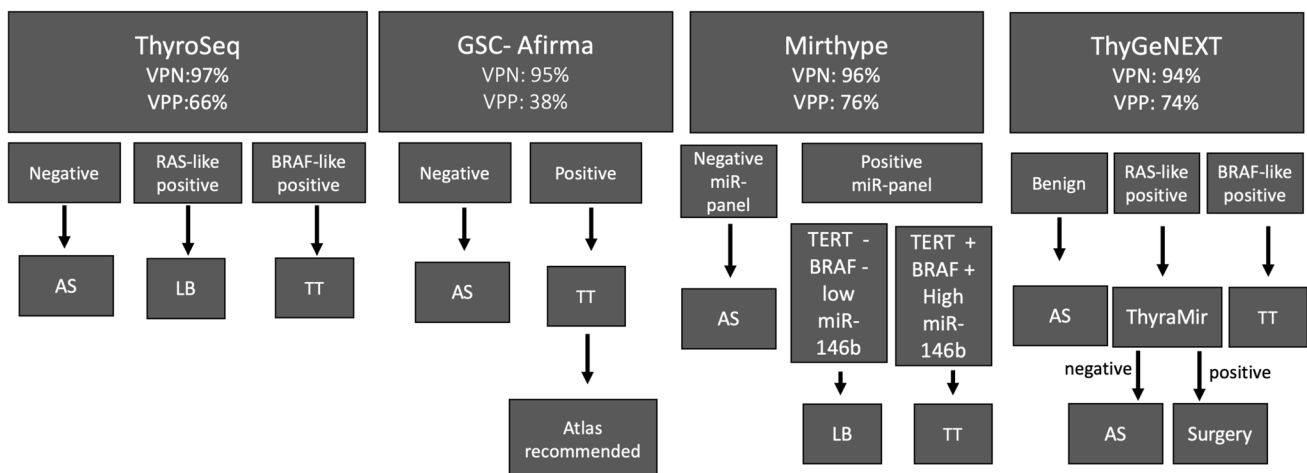

Fig. 2 Practical scheme to guide the management based on the finding of the molecular test (Schematic flowchart to guide the management according to the utilized molecular test. AS: active surveillance, LB: lobectomy; TT: total Thyroidectomy}

BRAF V600E	RET mutation RET rearrengements	NTRK
Dabrafenib Trametinib	Selpercatinib Pralsetinib	Larotrectinib Entrectinib

Fig. 3 Target mutation and the respective available FDA approved target therapy

Vemurafenib, although not yet approved by the FDA as a drug for thyroid treatment, also targets BRAF as a therapeutic target. Some preliminary studies have shown efficacy with few side effects, but it still requires phase 3 trials [51]. Similarly, Encorafenib is another BRAF inhibitor that is not yet FDA-approved but has shown promising results in melanoma and colorectal cancer, and has a longer half-life.

15 RET inhibitors

The RET mutation or rearrangements of the RET gene can be responsible for the oncogenesis of various types of thyroid cancer, including medullary carcinomas as well as DTC, including in children. Therefore, it also becomes a highly interesting therapeutic target.

Since 2020, Selpercatinib and Pralsetinib are the two FDA-approved drugs targeting RET [54, 55].

16 NTRK inhibitors

Tumors driven by NTRK fusions are rarer than other mutations, but they are of particular interest due to their prevalence in the pediatric population, reaching up to 25% [56]. Typically, tumors with these driver fusions can lead to central nervous system metastases.

Larotrectinib and Entrectinib are the FDA-approved drugs that have shown promising results in the progression of tumors harboring these rearrangements, including those with CNS metastases [51].

17 Conclusion

With precision medicine, molecular markers now play a role throughout the patient's journey, starting from the moment of diagnosis, where they have an important and well-established role in indeterminate cytology. They are also gaining more prominence in determining the extent of surgery and, ultimately, in the decision-making for targeted therapies. With the personalized approach to medicine and advancement in NGS techniques, molecular markers will continue to gain importance and actively participate in management decisions. The main molecular tests available worldwide today are capable of providing high sensitivity and specificity at each stage of this journey. Therefore, it is essential for clinicians to be familiar with the key molecular alterations, be aware of the available molecular tests, and, most importantly, know how to use them appropriately for a qualified clinical practice.

Declarations

Conflict of interest No conflict of interest.

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