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REVIEW ARTICLE

Molecular markers in well-differentiated thyroid cancer

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Abstract

Purpose Thyroid nodules are of common occurrence in the general population. About a fourth of these nodules are indeterminate on aspiration cytology placing many a patient at risk of unwanted surgery. The purpose of this review is to discuss various molecular markers described to date and place their role in proper perspective. This review covers the fundamental role of the signaling pathways and genetic changes involved in thyroid carcinogenesis. The current literature on the prognostic significance of these markers is also described.

Methods PubMed was used to search relevant articles. The key terms "thyroid nodules", "thyroid cancer papillary", "carcinoma papillary follicular", "carcinoma papillary", "adenocarcinoma follicular" were searched in MeSH, and "molecular markers", "molecular testing", mutation, BRAF, RAS, RET/PTC, PAX 8, miRNA, NIFTP in title and abstract fields. Multiple combinations were done and a group of experts in the subject from the International Head and Neck Scientific Group extracted the relevant articles and formulated the review.

Results There has been considerable progress in the understanding of thyroid carcinogenesis and the emergence of numerous molecular markers in the recent years with potential to be used in the diagnostic algorithm of these nodules. However, their precise role in routine clinical practice continues to be a contentious issue. Majority of the studies in this context are retrospective and impact of these mutations is not independent of other prognostic factors making the interpretation difficult.

Conclusion The prevalence of these mutations in thyroid nodule is high and it is a continuously evolving field. Clinicians should stay informed as recommendation on the use of these markers is expected to evolve.

Keywords Thyroid neoplasm/diagnosis · Thyroid neoplasm/genetics · Carcinoma, papillary · Adenocarcinoma, follicular · NIFTP · miRNA

This article was written by members and invitees of the International Head and Neck Scientific Group (http://www.IHNSG.com).

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Introduction

The prevalence of thyroid nodules ranges from 4–10% in the general population. This prevalence substantially increases to 50–70% when ultrasound is used for detection [1]. There has been a worldwide thyroid epidemic with 470,000 women and 90,000 men being over-diagnosed during the last 2 decades [2]. Though the disease-specific mortality of thyroid cancer is low, unwarranted or inadequate surgery is associated with increased morbidity making proper management important. Guidelines recommend fine needle aspiration cytology (FNA) for assessment of thyroid nodules [3]. However, it has limitations including indeterminate results and high inter-observer variability. The FNA is reported as indeterminate in around 25% of cases where the rate of malignancy ranges from 14 to 48% [4, 5]. It is important to identify malignant cases accurately to avoid unnecessary surgeries in those with benign nodules and target necessary therapy at those who require it.

The extent of surgery required for early tumours is controversial [3]. In part, this is due to an inability to determine the precise biology of tumours based on ultrasound and cytological features. There is a plausible need to take into consideration the biological aggressiveness of the tumour to refine surgical planning.

Recent years have witnessed considerable progress in the understanding of molecular changes underlying thyroid carcinogenesis. Molecular analysis is an emerging field and may enhance the prediction of both benignity and malignancy in thyroid cytology samples, increasing the total accuracy in cases when cytology is combined with molecular testing. Furthermore, molecular analysis is now formally included as an option for further evaluation of indeterminate cytology [3]. Despite the potential value in adults, molecular testing is not recommended in the pediatric population [6].

This review focuses on the current knowledge about molecular pathogenesis and markers of Differentiated Thyroid Cancer (DTC); their diagnostic and prognostic significance. We look at the fundamental role of signalling pathways involved in thyroid cancer and the genetic–epigenetic changes at the core of these pathways. Further, we consider molecular markers and tests developed thereof, their current role and evidence in diagnosis and prognosis in thyroid cancers.

Molecular pathogenesis of differentiated thyroid cancers

A large body of research in recent years has helped to improve our knowledge about molecular pathogenesis of thyroid cancers. This work has led to identification of specific molecular derangements and putative molecular pathways. More recently, The Thyroid Cancer Genome Atlas (TCGA) has performed a comprehensive analysis of Papillary Thyroid cancers (PTC) using multiple techniques including next generation sequencing. This has led to identification of specific abnormalities of significance in 96% of tumours [7].

BRAF mutations and mitogen-activated protein kinase (MAPK) pathway

BRAF is a serine–threonine kinase, belonging to family of RAF proteins, which are effectors of MAPK pathway. The MAPK is an intracellular signalling pathway that has a core function in cell growth, proliferation, apoptosis and differentiation; *BRAF* alterations potently activate this pathway.

One of the most important gene mutations that play an important role in thyroid carcinogenesis and that has been most extensively studied involves *BRAF* [8]. A point mutation (T1799A) causes V600E amino acid substitution in the BRAF protein, which is one of the common mutations that constitutively activate serine–threonine kinase. The frequency of this point mutation can be as high as 90% but is seen on an average in 45% of PTC [9]. *BRAF* mutations are not identified in benign thyroid nodules.

RAS mutations and phosphoinositide 3 kinase-AKT (PI3-AKT) pathway

RAS mutations are the second most common mutations in DTCs [9]. *RAS* in active state is bound to GTP and has intrinsic GTPase activity that converts GTP to GDP thus inactivating it. Mutation results in loss of this GTPase activity leading to its constitutive activation. Of the 3 isoforms—*HRAS, KRAS, NRAS*, the most common mutations in thyroid cancers are in *NRAS*. Though *RAS* can activate both the MAPK and PI3-AKT pathway, the latter appears to be preferentially activated by the mutated *RAS* [9]. The PI3K-AKT pathway has a predominant role in follicular patterned neoplasm including follicular thyroid carcinoma (FTC), wherein it promotes invasiveness and metastases. However, this appears to be an early mutational event having been identified in a portion of follicular adenomas also.

RET/PTC rearrangement

The *RET* proto-oncogene encodes a cell membrane receptor tyrosine kinase. *RET* is highly expressed in parafollicular C cells. It is usually not expressed in follicular cells, but it can be activated by chromosomal rearrangement: the *RET*/*PTC* translocation. This occurs due to genetic recombination between 3′ tyrosine kinase of *RET* and 5′ portion

of a partner gene. The translocation constitutively activates tyrosine kinase activity of *RET*. *RET/PTC* activates both the MAPK and PI3-AKT pathways [9]. There are more than 10 types of this translocation, and the most common are *RET/PTC1* and *RET/PTC3* [9–11].

Together *RET/PTC1* and *RET/PTC3* rearrangements account over 80% of DTC [12, 13]. *RET/PTC1* results in better differentiated PTC; whereas *RET/PTC3* is more specifically observed in radiation-induced solid-follicular PTC [14]. *RET/PTC* related subtypes show more regional metastasis [15]. *RET/PTC* carcinogenesis follows increased expression and phosphorylation of epidermal growth factor receptor (EGFR) and can be potentially targeted by EGFR related tyrosine kinase inhibitors (TKIs) [16].

PAX8/PPARγ rearrangement

PAX8/PPARγ rearrangement is caused by (2;3)(q13;p25) translocation that leads to fusion between the *PAX8* gene and the peroxisome proliferator-activated receptor-γ (*PPARγ*) gene [17]. *PAX8/PPARγ* has an inactivating effect on the wild-type tumour suppressor *PPARγ* and also transactivates certain *PAX8* responsive genes [9]. This translocation occurs in about 30–60% of FTC [9, 18, 19] and also in 38% of follicular variant of papillary thyroid cancer (FVPTC) [19]. There is no overlap between *PAX8/PPARγ* and *RAS* mutations in the same tumour [20], suggesting that FTCs may develop via 2 distinct pathways.

Other genetic alterations

Other genetic alterations are also involved in thyroid carcinogenesis. Mutations in the tumour suppressor gene *PTEN* activates the PI3K-AKT pathway, and forms the genetic basis of thyroid cancer in Cowden's disease [9]. *TRK* rearrangements may be found in less than 5% of PTC [20]. An important recent discovery is of the human Telomerase Reverse Transcriptase *(TERT)* promoter gene mutations. This represents a newly discovered mechanism, by which cells acquire telomerase activity; these mutations have been found in aggressive thyroid cancers [21]. Less commonly *p53, APC* mutations (FAP), *ALK* translocations, *EIF1AX*, etc. have also been described [22].

MicroRNAs (miRNAs) are short endogenous non-coding RNAs which regulate gene expression at mRNA post-transcriptional level in proliferation, apoptosis, and differentiation. Deregulation of miRNA expression is believed to be an important regulator of tumour development and progression [23]. Expression of miRNAs differs between PTC and benign thyroid lesions which may have a diagnostic implication in thyroid FNA and surgical pathology. In PTCs, there is a significant increase in miRNA (miR)-221, -222 and -181b as compared to normal thyroid. In particular, miR 221 over expression may have a critical role in thyroid cancers [24]. In a study, it was observed the seven miRNAs: miR-187, miR-221, miR-222, miR-146b, miR-155, miR-224 AND miR-197 was most consistently over expressed in follicular cell-derived carcinomas. However, their expression varied significantly between individual tumour types. When at least one miRNA was over expressed more than twofold, the sensitivity, specificity, and accuracy for malignancy detection were 100, 94, and 95% respectively [25].

Molecular markers for diagnosis

Understanding the molecular signatures of thyroid neoplasia has opened up new avenues to diagnosis of thyroid malignancies beyond cytologic classification; this is likely to have potential in nodules which are indeterminate on FNA. The Bethesda Reporting System for Thyroid Cytology (TBRSTC) is widely employed for reporting the outcome of FNA [26]. In 15–30% cases, FNA cannot ascertain if the nodule is benign or malignant/ suspiciously malignant [27]. This includes Bethesda categories III, Atypia of Unknown Significance (AUS)/Follicular Lesion of Unknown Significance (FLUS) and IV, Follicular Neoplasm (FN). The risk of malignancy (ROM) in these categories varies from 5–15% (category III) to 15–30% (category IV) [26]. This represents a challenge to physicians, since this ROM is too high to ignore. Performing 'diagnostic surgery' to obtain histopathology would be unnecessary in 70–80% of cases. In addition, there would be a need to perform an additional 'completion surgery' in nodules which are found to be 'high-risk' on pathology. A large body of work has focussed on the application of molecular alterations detection for improving the pre-operative diagnosis of thyroid cancers.

Recently non-invasive encapsulated follicular variant of PTC (FVPTC) has been classified as non-cancer entity with a revised nomenclature, non-invasive follicular neoplasm with papillary-like nuclei (NIFTP) [28, 29]. This poses a challenge to diagnostic accuracy of thyroid nodules further [30]. Studies have shown that these tumours are mainly classified as indeterminate on FNA [31]. With the reclassification of these tumours as non-malignant, there is a drop in ROM in the indeterminate categories. In a recent update taking this into account the ROM has shown a shift for category III from 10–30 to 6–18%, category IV from 25–40 to 10–40% and category V from 50–75 to 45–60% [32]. Although this shift in nomenclature results in a drop in ROM, as NIFTP is still viewed as a neoplasm with frequent molecular alterations in *RAS*, lobectomy is still indicated for diagnosis and treatment.

To date, molecular elucidation in differentiated thyroid cancer (DTC) has found its main application in diagnosis; with several retrospective and prospective studies showing that the diagnostic accuracy of FNA can be significantly

improved using molecular detection. We now review the genetic alterations that have shown diagnostic potential in FNA samples.

BRAF

BRAF V600E mutation has been the most extensively studied biomarker in FNA specimens. *BRAF* mutations are the most common genetic anomaly in papillary thyroid cancer (PTC), and are seen in about 45% of PTCs [9]. These are more common in sporadic PTCs, than in pediatric and radiation-induced PTCs [13]. *BRAF* mutations are rare in follicular thyroid cancer (FTC) and do not occur in benign thyroid nodules. Hence *BRAF* V600E mutation is a reasonably specific marker for PTC; however, this is the same group of patients where cytologic classification excels [12]. A review of 18 studies looking at *BRAF* testing in thyroid FNA samples showed that the rate of malignancy in *BRAF*-positive nodules was 99.8% [33]. Moreover, it has been reported that 15%-39% of *BRAF*-positive FNA samples were indeterminate or non-diagnostic; thus proving diagnostic utility of *BRAF* in indeterminate cytology. However, this utility of *BRAF* as a diagnostic marker for indeterminate nodules is limited by its low sensitivity for malignancy [34]. Further *BRAF* V600E is infrequent in FNA of indeterminate nodules (< 10%) which are typically follicular patterned neoplasms [35]. Nevertheless, irrespective of Bethesda category, when FNA testing reveals *BRAF* V600E, a diagnosis of thyroid cancer should be strongly suspected, though rare false positive cases have been reported [36]. Although *BRAF* V600E is the most commonly studied mutation, *BRAF* 599 and 601 have also been described which are seen in follicular patterned tumours. Translocations involving *BRAF* have also been documented.

RAS

RAS mutations are seen in 40–50% of FTC [20, 37] and in 10–20% of PTC [20]; most of which are FVPTC [38, 39]. However, *RAS* mutations are not specific for malignancy, and are also seen in 20–40% of follicular adenomas and are the predominant mutations in NIFTP [20]. This limits the utility of *RAS* as a sole diagnostic marker of malignancy.

In a cohort of 199 thyroid carcinomas that underwent molecular characterisation 27 were *RAS* mutation positive. Of these 20 were FVPTC, of which 16 would now be called NIFTP. Additionally, 59% of *RAS* mutation positive carcinomas would now be classified as NIFTP [40]. These tumours are reported as suspicious by Afirma and constitute a significant proportion of carcinomas detected [41]. With the recent reclassification of NIFTP [28, 29] it is likely that the specificity of this molecular marker for carcinoma may decline. Nevertheless, it is argued that though not 100% specific, *RAS* detection implies a neoplasm for which surgical management, lobectomy only allows for further diagnosis and definitive treatment. The risk of malignancy in *RAS* FNA nodules awaits recalibration for incidence of true carcinomas/malignancies versus neoplasm (adenoma or NIFTP) secondary to the recent shift in classification.

RET/PTC

RET/*PTC* translocations are found in 15%-20% of sporadic adult PTC, but only in 6.8% in data from TCGA [7, 33, 36, 42]. They are more common in radiation-induced and pediatric PTCs [10, 13, 43]. *RET*/*PTC1* is the most common rearrangement type, seen in 60–70% of all cases [20]. In a retrospective study *RET*/*PTC* identified malignancy in 60% of indeterminate nodules with 0% false positive rate [44]. The results confirm that *RET*/*PTC* is a highly specific biomarker for the diagnosis of PTC. However, *RET*/*PTC* rearrangements in benign nodules remains debated [45]. The presence of these translocations implies risk of malignancy warranting further diagnostic characterization.

Panel testing

From the foregoing discussion, it can be inferred that the use of individual diagnostic markers has insufficient sensitivity and diagnostic accuracy. Hence efforts were directed towards the development of a panel of molecular markers and alterations for evaluation in FNAs. One initial approach was the development of a panel of mutations including *BRAF, RAS, RET*/*PTC* and *PAX8*/*PPARγ*. This panel was first studied in 2 independent studies, and then in a large prospective study of indeterminate FNAs. The rate of malignancy in this study was 24%, which is important to note as the rate of malignancy in the population being tested will affect the subsequent determinations. For indeterminate FNA, the panel had sensitivity 61%, negative predictive value (NPV) 89%, specificity 98%, and positive predictive value (PPV) 89% [35]. Thus, the main impact of this panel was a rule in test by which the PPV and specificity for malignancy allowed for triaging patients to surgery when a mutation was identified.

Another approach was based on the measurement of mRNA expression. This gene expression classifier (GEC) yielded a high NPV (93%) that reduced the ROM to about 5% in GEC benign nodules similar to the risk in a benign cytology FNA [46]. Thus, utilizing this method as 'rule out' could aid in triaging patients at low-risk for malignancy, without proceeding to surgical evaluation. Moreover, the advancement of next generation sequencing to allow for numerous loci analysis on minimal quantity of nucleic acids, further enhanced integrations of thyroid FNAs to methods currently clinically available.

Concept of 'rule in' and 'rule out'

The choice of Molecular Test/Panel to be used broadly falls into two groups: (1) whether a test can 'rule in' malignancy (the likelihood that the nodule is malignant); or 'rule out' malignancy (the likelihood that the nodule is benign); and (2) depends on the performance factors of each test, the sensitivity, specificity, PPV and NPV.

A highly sensitive test is one which is usually positive in the presence of disease. Thus, a negative result of a highly sensitive test is associated with near surety of the absence of disease and conclusively rules out a condition. A highly specific test is one which is usually negative in the absence of disease. A positive result of a highly specific test is valuable in ruling in the presence of disease. In addition, the negative and positive predictive values of a test are directly proportional to its sensitivity and specificity, respectively, as well as to the prevalence of malignancy in the population being tested.

Of all the indeterminate nodules that undergo diagnostic surgery, two-thirds prove to be benign [4]. Thus, for the AUS, and FLUS and follicular neoplasm (FN) or Hurthle cell neoplasm categories (Bethesda III and IV), which harbour a 5–30% risk of malignancy, an ideal molecular test to "rule out" malignancy would be one with high sensitivity and high NPV. Similarly, with regards to the suspicious for thyroid malignancy (Bethesda V) category, with a 60–75% risk of malignancy, an ideal molecular test to "rule in" would be one with high specificity and high PPV. However, as no test is entirely sensitive, and Bethesda V has a high-rate of malignancy, this cohort warrants surgical evaluation of the nodule as ancillary tests cannot definitively exclude malignancy.

Commercially available molecular tests

There are a number of commercially available clinical tests to help further risk stratify indeterminate nodules to predict the presence or absence of malignancy (Table 1). Methods that classically test for the presence of gene point mutations are used as 'rule in test': gene point mutations (*BRAF* or *RAS*) or gene rearrangements (*RET*/*PTC, PAX8*/*PPARγ*). The Afirma GEC that checks for RNA expression is a classic example of 'rule out test'.

Afirma gene expression classifier

This is a microarray based test to analyse mRNA expression of 167 genes. A multicentre trial of GEC showed a high NPV of 95% and 94% for nodules in the AUS/FLUS and FN/SFN (suspicious for follicular neoplasm) categories (Bethesda categories III and IV), respectively [46]. Therefore, the ROM in these categories when the Afirma GEC test result indicated "benign nature" ranged from 5 to 6% and closely approached the NPV in thyroid FNAs diagnosed as benign. The GEC, however, has a low NPV value of 85% in Bethesda V 'Suspicious for malignancy' (thus representing a risk of cancer of 15%). Hence the test is recommended only for categories III-IV. Further, the GEC has a low PPV value (37–38%) [46]. This test is therefore useful as a 'rule out' test. If the diagnosis is 'benign' in the indeterminate category, the patient could be followed up clinically with no need for surgery. However, if the diagnosis is 'suspicious', the diagnosis remains indeterminate [47].

miRInform test

This test is based on analysis panel of 4 DNA mutations (*BRAF, RAS, HRAS*, and *NRAS* point mutations) and 3 RNA translocation fusion markers (*RET*/*PTC1, RET*/*PTC3*, and *PAX8*/*PPARγ*) [47]. In a validation study the mutation positivity rate was significantly higher in the malignant cases (56%) with highest rate reported in classical PTC (79%) [48]. However, this test lacks sensitivity; it can therefore be used as a 'rule in' test to confirm malignancy in indeterminate or suspicious nodules on cytology.

ThyGenX test

This is a modified and currently offered version of the miRInform test. It uses next generation sequencing to identify more than 100 genetic alterations in 8 genes [47]. The test is applicable to only Bethesda Categories III-IV. Recently, a new test called ThraMIR is offered by the parent company,

Table 1 Overview of molecular diagnostic panel tests for thyroid FNA [47]

	Afirma	ThyGenx	ThyroMIR	Thyroseq
Characteristics	High NPV and low PPV (rule out)	High PPV and low NPV (rule in)	High NPV and PPV when combined with ThyGenx (combined)	High NPV and PPV (combined)
Methodology	mRNA gene expression	Multiplex PCR	MicroRNA expression	Next generation sequencing
Test results	Benign/suspicious[a]	Specific gene mutation/translocation	Negative/positive	Specific gene mutation/translocation

[a]Afirma reporting is as benign profile versus noting a nodule as "suspicious" when it does not fall into the low-risk/benign group

which is meant to be used in conjunction with ThyGenX when the result is negative. Using a combination of ThyGenX and ThyraMIR yielded a NPV and PPV of 94 and 74%. When both test results were negative, the residual risk of cancer was 6% [49].

ThyroSeq test

This is a next generation sequencing based gene mutation and fusion panel, targeting 284 mutational hot spots in 12 genes [50]. The assay identified mutations in 70% of PTC, 83% of FVPTC, 78% of FTC and 39% of oncocytic follicular carcinoma. The test has high specificity and hence this can potentially be used as a 'rule in' test. A more recent version ThyroSeq v2 includes a more exhaustive panel of DNA and RNA alterations [point mutations in 13 genes and for 42 types of gene fusions: point mutations (*AKT1, BRAF, NRAS, HRAS, KRAS, PTEN, TP53, TSHR, GNAS, CTNNB1, RET, PIK3CA*, Primers for detecting mutations at the cytosine-to-thymine 228 and 250 hotspots of the *TERT* gene promoter) and gene fusions (38 types of *RET* fusion genes including *BRAF, NTRK1, NTRK3, ALK, PPARG, THADA* to different partners)] and 8 genes as part of RNA panel to estimate the quantity of cells (*PGK1* gene, *TG, TTF1, NIS, KRT7, CALCA, PTH, KRT20*). A study using this version yielded a PPV of 83% and NPV of 96% [51] thus the ThyroSeq v2 may be used as both a 'rule out' and 'rule in' test. The latest version is ThyroSeq v3 which analyses 112 genes for point mutations, fusions, copy number changes, and expression levels that had been developed using a training set of 238 operated thyroid nodules. This was then validated on 175 FNA samples with known surgical follow-up. It showed a high sensitivity of 93.9%, specificity of 89.4% and accuracy of 92.1% on the tissue training set. The sensitivity was 98.0%, specificity 81.8%, and accuracy 90.9% in FNA validation set [52].

Rosetta GX assay

This is a molecular microRNA-based assay which differentiates between benign and malignant nodule using reverse transcriptase-polymerase chain reaction (RT-PCR). In a study of over 800 FNA the results showed that the assay can be run on FNA slides with as little as 1% thyroid epithelial cells and with only 5 ng of RNA [53]. The level of concordance was high between the laboratories and for the slides created from the same FNA pass. In another multicentric validation study on 189 samples, NPV, sensitivity and specificity was 91, 85 and 72%, respectively [54].

The new American Thyroid Association (ATA) Guidelines [3] recommend patient counselling about the potential benefits and limitations of molecular testing. The guidelines also state that since the long-term outcome data on use of molecular testing is still insufficient, it is debatable whether molecular testing should be used in routine practice for indeterminate cytology thyroid nodules.

Molecular markers for prognosis

Patient and tumour factors including age, tumour size, and presence and degree of extra thyroidal extension, number, size, location, extra nodal extension and distant metastases have been used to predict the risk of recurrence and mortality in DTC. Unfortunately, the majority of these are unavailable preoperatively. Postoperative ultrasound and thyroglobulin estimation are also used in clinical risk prediction [3]. More recently work has focused on molecular markers in predicting outcome for patients with thyroid cancer. Incorporation of these molecular markers into systems for risk stratification, which are available preoperatively, appears conceptually attractive in tailoring patient management in terms of initial extent of surgery, adjuvant therapy and postoperative medical management.

BRAF

Forty-eight to eighty percent of PTCs are associated with mutations. The most common mutation that occurs and studied extensively for its prognostic potential is *BRAF*, others being *RAS, RET/PTC* rearrangement and *TERT* [55, 56]. In a recently published meta-analysis, the *BRAF* mutation was present in 41.2% of all PTCs [57]. The prevalence of *BRAF* mutation is higher in conventional PTC (51%) when compared to FVPTC (24.1%) and follicular carcinoma (1.4%) [58]. There is geographical variation in the prevalence of *BRAF* mutation with a relatively higher prevalence in Asia [59].

BRAF is the most common mutation in PTC and most extensively studied for prognostication. *BRAF*V600E usually portends poor outcomes in PTC, and is associated with aggressive pathological features, increased rates of recurrence, loss of radioiodine avidity and treatment failures [60, 61]. In a multicentre study of 219 PTC, *BRAF* mutation was found to be significantly associated with extrathyroidal extension, lymph node metastasis, and advanced tumour stage. The mutation was also an independent predictor of recurrence. The mutation was associated with loss of radioiodine avidity and treatment failure in recurrent disease [61]. A meta-analysis of over 5000 patients also showed that the *BRAF* mutation was associated not only with an increased odds ratio of extrathyroidal extension, lymph node metastasis and advanced stage but also with a 2.14-fold increased risk of disease recurrence and persistence [56]. In another study 46% of those patients with central compartment lymph node metastasis had *BRAF* positivity, this

being the only independent predictor of central compartment metastasis [62]. In a retrospective study of 1849 patients, cancer-related mortality was significantly higher in *BRAF* positive patients; however, the association was not independent of other tumour features [63]. However, majority of these studies are retrospective and the impact of *BRAF* positivity in some studies is not independent of other tumour features making the interpretation difficult.

These mutations are associated with aggressive tumour behaviour and poorer outcomes even in conventionally low-risk patients and papillary thyroid micro carcinoma (PTMC). In a comparative study of 1150 patients, it was seen that the frequency of a *BRAF* mutation was similar in PTMC and PTC (65.6% vs. 67.2%) [64]. A *BRAF* mutation was associated with signs of higher aggressiveness, multifocality, extrathyroidal invasion, lateral neck compartment lymph node metastasis and advanced tumour stages III and IV in PTMC [65, 66]. A meta-analysis of over 3000 patients concluded that *BRAF* positive PTMC is associated with tumour multifocality, extrathyroidal extension, lymph node metastasis and advanced stage [67]. *BRAF* mutations in low-risk cancer [intrathyroid tumours and without metastasis (T1-2N0M0)] was a poor prognostic factor for persistence of disease and these patients required radioiodine courses of a higher dose to obtain disease free status [68]. In another retrospective multicentre study, recurrence rates were significantly higher in *BRAF* mutation positive PTC. This significant association of *BRAF* mutation positivity with recurrence was also seen in conventionally low-risk disease stage, micro PTC and within various subtypes [69]. Therefore, there may also be an argument for treatment intensification for these low-risk patients with microcarcinomas who may otherwise be considered for observation in some settings.

Studies have shown that the detection of *BRAF* mutation had an independent correlation with worse outcome on multivariate analysis and mutation positive patients had lower percentage of survivors [70]. However, the long term impact of the mutation on survival has been challenged. At the median follow-up of 8 years there was no significant relationship between *BRAF* mutation and recurrence-free survival and disease-specific survival (DSS) in another recent study [71]. A large meta-analysis reported that *BRAF* mutations were significantly associated with poor disease-free survival (DFS) and DSS at short/ medium follow-up (five or less than 5 years), however, this impact was lost at long term follow up of over 5 years [57].

The clinical application of *BRAF*V600E as prognostic marker is impaired by its low specificity. A meta-analysis showed an acceptable sensitivity (65%), but a poor specificity for the prediction of recurrent disease with a PPV of only 25%. Even though the mutation is associated with aggressive clinicopathological features, impact on survival in long term is not well established. Thus, the current role of mutated *BRAF* for risk stratification of PTC is limited. It is unlikely to be used in isolation, but only in a multivariable context, combined with other prognostic features [72]. While *BRAF* may be associated with increased local recurrence as recognized by the ATA risk stratification [3] its role as an independent factor remains limited and its co-role with *TERT* promoter mutation may be the link to truly aggressive PTC, however prospective studies are needed.

RAS

RAS mutations are associated with tumour dedifferentiation and less favourable prognosis [20, 33, 73, 74]. Some studies have found a significant correlation between *RAS* mutation and metastatic behaviour (bone metastases, in particular) and poor survival of follicular and papillary carcinomas [73, 74]. However, *RAS* mutations may also be seen in encapsulated FVPTC, which is now considered non-cancer entity. Thus, *RAS* mutation in a thyroid nodule 'provides strong evidence for neoplasia, although it does not establish the diagnosis of malignancy'. At times, these mutations are found in adenomas; hence it is possible that *RAS* mutated follicular adenomas are precursor lesions for FTC and FVPTC [20, 33].

Others

Clinically, *RET/PTC* positive PTC patients are younger and have classic papillary pathology and a high propensity of nodal metastases [15]. The prognostic implication of *RET/PTC* positive tumours are not clear. There is evidence that *RET/PTC1* is associated with more favourable behaviour in PTC; [75, 76] which contrasts with evidence that *RET/PTC3* may portend dedifferentiation and more aggressive behaviour [76].

Clinically *PAX8/PPARγ* tumours are seen in younger patients, which are of smaller size with more frequent vascular invasion. Detection of *PAX8/PPARγ* in a follicular lesion should prompt the pathologist to perform a thorough search for vascular or capsular invasion [20].

Published studies have shown that *TERT* mutations are present in a small number of PTCs; however, these are subclonal [77]. In a meta-analysis, *BRAF* mutations concomitant with *TERT* mutations were present in 6.2% and *TERT* alone in 4.2% of cases. *TERT* promoter mutations were associated with poor DFS and DSS and have been shown to be an independent predictor of recurrence and mortality [57]. *TERT* mutations were significantly associated with unfavourable survival both in short/ medium and long term follow-up. However, the majority of studies were retrospective and large prospective studies are needed.

Thus, the use of molecular markers for prognosis is plagued by a number of factors including the lack of

specificity, variable reports regarding prognosis, and limited clinical utility. The use of molecular markers for prognosis to date has not found routine application in clinical practice. Current guidelines do not recommend the use of these markers for initial risk stratification. However, *BRAF* and *TERT* promoter mutations are included in risk stratification for recurrence [3].

Another important factor of all the molecular tests is their high cost ($1675–$4875) [47]. For these to be commercially viable, results obtained should be able to offset the cost of treatment had the test not been offered. Yip et al. reported cost savings if the test was less than $870. They concluded that molecular testing of indeterminate nodules can allow cost savings and improve patient care by providing an indication for total thyroidectomy when testing is positive [78]. However, these calculations may not hold true for regions outside the North America. In developing countries, the cost of the surgery may itself be lesser than the cost of the test. Further, the insurance coverage and a ceiling for such molecular tests are unlikely to be uniform.

Conclusion

The role of molecular markers in the management of patients with thyroid nodules and cancer is evolving. Although such tests have a potential role both in diagnosis and prognostication, the American Association of Clinical Endocrinologists (AACE) guidelines state that, "At present, molecular testing is meant to complement and not replace clinical judgment, sonographic assessment, and visual cytopathology interpretation. As molecular testing is new and advances in the field are regularly occurring, clinicians need to stay informed as recommendations for use within practice are expected to evolve" [79].

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Compliance with ethical standards

Conflict of interest The authors declare that there is no competing interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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