

Molecular testing in the diagnosis of differentiated thyroid carcinomas

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Abstract: Different genetic mutations and other molecular alterations in papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC) can be detected in fine-needle aspiration (FNA) of thyroid nodules, and can be used successfully to ameliorate cancer diagnosis and management of patients with thyroid nodules. The greatest experience has been obtained with the diagnostic use of BRAF mutation that is strongly specific for malignancy when detected using well-validated techniques. The strongest diagnostic result can be obtained testing FNA samples for a panel of mutations that typically involve TERT, BRAF, PAX8/PPAR γ , RAS, and RET/PTC. Finding any of these mutations in a thyroid nodule provides strong indication for malignancy and helps to refine clinical management for a significant proportion of patients with indeterminate cytology. The use of molecular markers, as TERT, BRAF, PAX8/PPAR γ , RAS, and RET/PTC, may be considered for patients with indeterminate FNA cytology (FNAC) to help guide management. In patients with indeterminate TIR3 FNA, the combination of precise molecular marker expression analysis with molecular mutations evaluations could ameliorate significantly the accuracy of cancer diagnosis. However other prospective studies are needed to identify more accurate molecular markers. Finally, the knowledge of these molecular pathways has permitted the development of new targeted therapies for aggressive TC.

Keywords: Fine-needle aspiration cytology (FNAC); follicular thyroid cancer (FTC); indeterminate thyroid nodules; molecular diagnostic; papillary thyroid cancer (PTC)

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Introduction

In the United States, thyroid cancer (TC) has the highest rate of growth among diagnosed tumors (1). TC accounts for 6% of women cancers and less than 3% of men cancers.

Even if most of TCs are sporadic (2), the nuclear disasters and the resulting radiation exposure (for instance,

Chernobyl, 1986), represent significant risk factors for TC development (3-5).

Papillary TCs (PTCs) and follicular TCs (FTCs) arise from follicular cells and represent the majority of TCs (90%), while medullary TCs arise from para-follicular C-cells, and are by far less common (5%). Thyroid malignancies span from the well-differentiated to the poorly

differentiated or undifferentiated (anaplastic) cancers (6).

Standard treatment for TC usually includes primary surgery (near-total or total thyroidectomy and lymph nodes dissection if necessary), ablation of the thyroid remnant with radioactive iodine (RAI) (based on the tumor stage) and thyroid-stimulating hormone (TSH) suppressive therapy (7).

Follow-up consists of neck ultrasonography (US), performed basally and after TSH-stimulated thyroglobulin assay (7-10).

Though TC has generally a good prognosis, 10% to 15% of patients with TC have recurrent disease, and about 5% will develop metastatic disease not responsive to RAI, and eventually will die from this disease (11-14).

For these reasons an early diagnosis of TC is needed in patients with thyroid nodules. US along with fine-needle aspiration (FNA) cytology (FNAC) are determinant to differentiate benign thyroid nodules from malignant nodules (7).

In order to improve FNAC accuracy in detecting malignancies, testing for oncogene mutations has been proposed (15,16), suggesting that it improves the performance of FNA diagnosis when it is performed in indeterminate cytology, supporting the clinician's decision making process in several settings of uncertain diagnosis. Testing for multiple mutations [BRAF, RAS, RET/PTC, PAX8/peroxisome proliferator-activated receptor (PPAR) γ] improves the performance and increases the specificity, but it does not increase the sensitivity as well (15).

For these reasons, molecular diagnostic is recommended in the 2015 revised American Thyroid Association management guidelines in the cases of patients with thyroid nodules and differentiated TC (DTC) with indeterminate cytology (17).

This study aims to review the potential advantages of assessing the gene-related cancer status on samples obtained with FNA, and their prognostic relevance.

Molecular pathways implicated in TC

Rearranged during transfection (RET)

RET is a proto-oncogene (located on 10q11.2) encoding a transmembrane protein harboring intracellularly a tyrosine kinase (TK). Activating RET mutations and rearrangements have been reported in human cancers (18). RET/PTC rearrangements have been identified in about 40% of adult sporadic PTC (19). The most common are RET/PTC1,

and RET/PTC3, often reported in microcarcinomas and benign thyroid lesions, leading to hypothesize that RET/PTC can be determinant for tumor initiation, but not progression (20-24).

RAF kinase

The BRAF kinase belongs to RAF family proteins; a BRAF mutation [substitution from valine to glutamate at residue 600 (V600E)] is present in approximately 45% of PTC, associated with tumor aggressiveness (24).

Vascular endothelial growth factor (VEGF)

TC aggressiveness and increased angiogenesis are correlated (25), and the expression of VEGF/VEGF receptor (VEGFR), platelet-derived growth-factor (PDGF)/PDGFR, fibroblast growth factor (FGF)/FGFR, epidermal growth factor (EGF)/EGFR, and hepatocyte growth factor (HGF)/c-Met (that stimulate or inhibit angiogenesis) is associated with clinical features of TC (26). VEGF and its principal receptor VEGFR-2 are overexpressed in DTC (27) and are implicated in tumor progression and aggressive behavior. Drugs that target VEGF pathway are indeed a therapeutic approach in TC (28).

Histone acetylation

On histones the acetylation of NH₂-terminal lysine controls cellular differentiation and biological behavior of neoplastic cells.

A more open chromatin configuration leads to an increase in gene transcription rate while a closed chromosomal configuration is linked to transcriptional repression (29).

The dysregulation of histone acetyltransferase or histone deacetylase (HDAC) activity is present in some neoplastic cells (30-33).

The HDAC inhibitors depsipeptide, vorinostat, belinostat, valproic acid, and panobinostat have an antineoplastic effect on TC cells (34).

Epidermal growth factor receptor (EGFR)

The EGFR cell-surface protein (ErbB-1; HER1 in humans) is part of the ErbB family of receptors (35), a subfamily of four closely related receptor TKs: EGFR (ErbB-1), HER2/c-neu (ErbB-2), HER3 (ErbB-3) and HER4 (ErbB-4).

Mutations can cause EGFR upregulation or over

activity, that have been correlated with various cancers, as lung cancer, anal cancer (36) and glioblastoma multiform (in which EGFRvIII results to be the most frequent mutation) (37).

Mutations, amplifications or misregulations of EGFR or of other family members are present in approximately 30% of all epithelial tumors, and EGFR is determinant in tumor progression and invasion in TC and is overexpressed in anaplastic TC (ATC) (38-40).

RAS

The RAS gene family members N-RAS, H-RAS, and K-RAS encode for intracellular G-proteins participating in the activation of intracellular signal pathways. Approximately 40–50% of FTCs and 10% of PTCs show RAS mutations (associated with a more aggressive TC behavior) (41).

TERT

The major part of normal cells does not have telomerase activity, while the upregulation of TERT transcription/activity is shown in 80–90% of malignant tumors. In different kinds of cancer, the presence of TERT promoter mutations, TERT mRNA expression and clinicopathological features are associated, even if the link between the occurrence of TERT promoter mutations and tumor aggressiveness is not known. Even the association between TERT promoter mutations and metastasis/survival has been reported in different cancers. In follicular cell-derived TC, such mutations are associated with worse prognostic features (age of patients, tumor size and stage) and distant metastases, less response to treatment and poor survival (42-44).

PAX8/peroxisome proliferator-activated receptor γ (PPAR γ) rearrangements

PAX8/PPAR γ rearrangements are detected in typical FTC (30–40%) and in Hürthle cell tumors (5%) (45), and their presence is linked to a good prognosis.

Tumors presenting PAX8/PPAR γ rearrangements do not have any RAS mutation, leading to hypothesize the coexistence of two separate mechanisms (PAX8/PPAR γ translocation or RAS oncogenic activation) that contribute to the development of FTC (45).

PAX8/PPAR γ rearrangements are detected in follicular adenomas (FAs; 2–10%), as in the follicular variant of PTC

(FVPTC) (46,47). Approximately 0–1% of PTCs have PAX8/PPAR γ translocations (46).

Molecular diagnosis of thyroid nodules

Thyroid nodules are very common, since 5% of women and 1% of men have palpable nodules in iodine-sufficient countries. However, 19–67% of randomly selected individuals have thyroid nodules detectable by US (7). TC occurs in 5–15% of thyroid nodules, depending on age, sex, radiation exposure history, family history, and other factors (48). Early diagnosis of malignant thyroid lesions allows to achieve a curative total thyroidectomy. For this reason, it is crucial to distinguish them among thyroid nodules, which are very often benign (49).

At present the combination of FNA with cytological evaluation is the common preoperative diagnostic method in TC, even though a number between 10% and 40% of cytological specimens still result indeterminate for malignancy (50).

Since 2008, the indeterminate cytology is classified in the following three subclasses: “follicular lesion of undetermined significance”, “follicular or oncocytic (Hürthle cell) neoplasm”, and “suspicious for malignancy”. These subclasses express the malignancy chance of 5–10%, 15–30%, and 50–75%, respectively (51).

Molecular testing of FNA biopsies (FNABs), overall for BRAF, and for other markers as RET/PTC, PAX8/PPAR γ , BRAF, and RAS, is possible and can significantly ameliorate the accuracy of the preoperative FNA diagnosis by cytology (52-62). In patient with indeterminate cytology, the investigation for genetic markers (PAX8/PPAR γ , BRAF, RAS, and RET/PTC) and protein markers (galectin-3) has demonstrated to improve the accuracy of pre-surgery diagnosis (56-58,63-65). Furthermore, the use of molecular markers (i.e., RET/PTC, BRAF, RAS, PAX8/PPAR γ or galectin-3) is now formally advised for indeterminate cytological samples in the “2009 Revised American Thyroid Association (ATA) Management Guidelines for Patients with Thyroid Nodules and Differentiated Thyroid Cancer” (Recommendation rating: C) (7).

BRAF

A number of studies (56-60,64,66-72) have reported that molecular testing for BRAF V600E in thyroid FNA samples significantly ameliorates the accuracy of cytological diagnosis of thyroid nodules. A recent meta-analysis of

18 studies reporting the results of BRAF testing in 2,766 thyroid FNA samples demonstrated that among 581 BRAF-positive cases, 580 were PTCs (73). Therefore just one BRAF-positive specimen collected from a thyroid nodule of resected gland, disclosed benign nature (74). Even if this case is recognized as a false-negative, the rate of malignancy in BRAF-positive nodules tested by FNA was 99.8%. Several studies have reported that 15–39% of BRAF-positive FNA samples were indeterminate or non-diagnostic by cytology, demonstrating that testing for BRAF is helpful in establishing a definitive diagnosis of cancer in nodules with indeterminate cytology (58,60,66,75-77).

Many authors have investigated the role of BRAF mutation in PTC; the data indicate that BRAF V600E mutation is an important prognostic molecular marker that improves FNAB diagnostic accuracy, but is complementary to cytology and US (61).

In fact, BRAF V600E mutation was significantly associated with malignant US features (solid composition, marked hypoechogenicity, irregular margin, taller-than-wide shape and the presence of microcalcification) (78), and also with poor prognostic factors and increased tumor size (79).

As regards FTC, detection of BRAF mutation had limited value, but in case of suspicious for malignancy FNABs can be helpful and results are associated with risk of extra-thyroidal extension and metastases after surgery (80).

A perspective study published in 2012 assessed the usefulness of US-guided FNAB in the diagnosis of nodules with/without clinical/US features suggestive for malignancy and to evaluate the contribution of BRAF V600E mutation analysis in the detection of DTC. Thyroid cytoaspirates from 2,421 nodules at least 4 mm in diameter were done in 1,856 patients submitted to cytological evaluation and bimolecular analysis.

Cytology had high positive predictive value and specificity for the diagnosis of malignant lesions. BRAF V600E mutation was reported in 115 samples, and 80 of them had also a diagnosis of PTC. The evaluation of the presence of a BRAF mutation significantly improved the diagnostic value of cytology, increasing FNAB diagnostic sensitivity for malignant nodules of about 28% (81).

Recently Howell *et al.* published a single-center, retrospective review (82) of all patients who had initial thyroidectomy for histologic PTC during 2010. The possible correlation between central compartment lymph node metastasis (CLNM) and available preoperative clinical parameters (as age, gender, tumor size, and BRAF mutation status) was evaluated. Considering the common clinical

preoperatively available parameters, the BRAF V600E mutation was the only independent predictor of CLNM in PTC and could be utilized to guide the extent of initial surgery (82).

In a recent paper, Kabaker *et al.* have confirmed that BRAF-positivity is correlated with the most known suspicious US findings, such as hypoechogenicity, ill-defined margins, calcifications, absent halo and taller-than-wide shape (83). Moreover, the positive predictive value raised to 82% when three or more US such features were present, while the negative predictive value (BRAF-negativity plus no US features) was 88% (83).

As up-to-date meta-analysis showed, BRAF mutation offers very high grade of specificity (97.9%) and positive predictive value (99.9%) with only 7 false positives described by 3 studies (74,84,85) of a total of 26, which included the evaluation of 2,800 malignant and benign thyroid neoplasms. Various techniques have been adopted to detect this marker without affecting the results, which remained constant (61-66,73-86) [from Rodrigues *et al.* (87)].

RET/PTC

Testing for RET/PTC rearrangements can be helpful for the diagnosis of TC. In thyroid FNA samples, RET/PTC detection can ameliorate the preoperative diagnosis of thyroid nodules, especially in samples resulting indeterminate by cytology or without a sufficient amount of cells for the cytological evaluation (54,60,66). In 2 prospective studies (58,64), all 16 thyroid nodules positive for RET/PTC were found to be PTCs after surgery, and many of those nodules were indeterminate by cytology. In another recent study (56) 5/6 nodules positive for RET/PTC in the FNA material were malignant, and 1 nodule was found to be benign after surgery.

Recently, Ferraz *et al.* have shown the feasibility of testing for RET/PTC rearrangements from routine FNA (88).

The conclusions of a more recent meta-analysis (87) are that the RET/PTC has shown average values of specificity (18%) and positive predictive value (87%).

RAS

The importance of RAS mutation detection lies in the fact that it represents a marker of FVPTC, which is most difficult to diagnose, in particular by FNAC. RAS mutations are the second most common mutation type detected in consecutive FNA samples from thyroid nodules (89).

A retrospective study on 341 patients with TC showed an association between malignancy and N-RAS mutation, as well as with tissue inhibitor of metalloproteinases-1 and FNAC result (90). In several prospective studies (58,64), RAS mutation had a 74% to 88% positive predictive value for malignancy. Importantly, RAS mutations were identified in tumors that were difficult to diagnose with only cytology, which is FVPTC and FTC. Some RAS-positive nodules were diagnosed as benign FAs, formally contributing to a false-positive rate. It is likely that RAS-positive FAs serve as precursor lesions for RAS-positive FTC and possibly FVPTC, and RAS mutation apparently predisposes the well-differentiated cancer to dedifferentiation and more aggressive behavior (91-96).

In a more recent meta-analysis (87), RAS mutations have displayed average values of specificity (23%) and positive predictive value (82%).

PAX8/PPAR γ

Detection of PAX8/PPAR γ rearrangement in a follicular lesion is not fully diagnostic for malignancy by itself, but it should encourage the pathologist to perform a complete search for vascular or capsular invasion. Although it may not be seen at first, the invasion is found in many PAX8/PPAR γ -positive follicular tumors after examination of the entire capsule in multiple histologic levels (97,98).

PAX8/PPAR γ rearrangement can be detected in thyroid FNA samples, and this correlates with the presence of malignancy, even if only few positive cases were reported so far in prospective studies (58,59).

Recently, Ferraz *et al.* have shown also the feasibility of testing for PAX8/PPAR γ rearrangements from routine FNA (88).

In a more recent meta-analysis (87), PAX8/PPAR γ mutations have shown average values of specificity (20%) and positive predictive value (100%).

Multiple testing

One prospective study, which compared cytology alone or combined with molecular testing for RET/PTC, BRAF, PAX8/PPAR γ , and RAS mutations, demonstrated to increase the FNA sensitivity for malignancy of thyroid nodules from 44% to 80%, respectively (58).

After surgery, 31/32 (97%) mutation-positive nodules were diagnosed as malignant, and 1/32 (3%) as a RAS-positive FA, suggesting the detection of each mutation can

be considered a good predictor of malignancy (58). After surgery, considering the indeterminate FNA samples, molecular testing permitted to identify 15/21 (71%) malignant nodules, indicating that molecular testing can ameliorate the diagnosis of indeterminate FNAs (58). Another paper showed that the sensitivity of malignant diagnosis in FNA rose from 60% to 90%, combining cytology with molecular testing for TRK, PPAR γ , BRAF, RAS, and RET mutations to cytology alone (64). Mutations were associated with cancers in 61/67 mutation-positive patients (91%) and with benign FA in 6/67 mutation-positive cases (9%), indicating as the previous study that the presence of a mutation could be a good predictor of malignancy. The usefulness of these markers in the pre-surgery setting is still under investigation.

Recently in a prospective study of Borrelli *et al.* (99) different consecutive FNA samples were assessed by independent cytological and molecular means. BRAF was mutated (by Sanger method) in the suspicious and malignant cases, but not in TIR1, TIR2, and TIR3 cases, suggesting that BRAF mutational analysis does not improve the cytological diagnosis.

A BRAF mutation was reported in only 3 patients (4.4%), analyzing the indeterminate nodules (cytological TIR3 cases) by the Easy BRAF kit, that is a very sensitive real-time-based method.

The above reported data indicate that the frequency of BRAF mutations in indeterminate cases was low also using a very sensitive method, depending on the expertise of the cytopathologist, on the accuracy to classify the TIR3 class, and on a selection bias due to the geographic region where the evaluation of the patients was conducted.

An attentive expression profiling analysis was performed to identify new potential diagnostic markers.

Thirty/68 cases of indeterminate nodules were submitted to surgery upon a clinical evaluation, and in 16/30 a histological diagnosis of benign lesions was done, and in 14 of malignant lesions. The malignancy rate in these 30 indeterminate FNA patients was high probably because the first group of patients undergoing surgery showed suspicious aspects with neck ultrasound (i.e., >8 mm; a hypoechoic, solid appearance; irregular or blurred margins; presence of microcalcification).

RT Profiler PCR arrays (100) were used for expression profiling analysis in all the 30 patients submitted to surgery. This technique permits to evaluate the expression profile of a specific panel of genes, and was used to analyze 84 genes participating in cell-matrix interactions, cell-cell adhesion,

and ECM remodeling. In tumors, between epithelial and microenvironment stroma cells, dynamic and reciprocal interactions, involving CAMs (i.e., integrin and CD44), ECM non-cellular components (i.e., thrombospondin 1 and FN1), and soluble cytokines exist. Furthermore, in human tumors the composition of CAMs and ECMs is changed and affects cell growth, survival, migration, adhesion, and other stromal and neoplastic cells features (101-103).

The following genes were identified: 6 genes upregulated in malignant nodules with respect to benign ones [“transforming growth factor, β -induced (TGFBI), cadherin 1, type 1 (CDH1), collagen, type I, $\alpha 1$ (COL1A1), catenin (cadherin-associated protein), $\alpha 1$, 102 kDa (CTNNA1), integrin $\alpha 3$ (ITGA3), and fibronectin 1 (FN1)”]; 6 genes strongly upregulated in FVPTCs *vs.* FAs [“integrin $\beta 2$ (ITGB2), FN1, connective tissue growth factor (CTGF), laminin $\gamma 1$ (LAMC1), catenin (cadherin-associated protein), $\delta 1$ (CTNND1), and integrin αV (ITGAV)”].

Moreover, 6 deregulated genes, {4 upregulated genes [secreted phosphoprotein 1 (SPP1), CTNNA1, TIMP metalloproteinase inhibitor 1 (TIMP1), and FN1] and 2 downregulated genes (ADAMTS1 and SELL)}, were reported in BRAF-mutated PTCs *vs.* wild type tumors.

These data agree with previously reported differences between malignant and benign thyroid nodules for molecules implicated in cell-cell adhesion, cytoskeletal reorganization, and cell matrix interactions, such as many collagens, catenin, FN1, or integrin (103-110).

Conclusions

Several genetic mutations occurring in PTCs and FTCs can be detected in FNA of thyroid nodules, and can be used to ameliorate cancer diagnosis and management of patients with thyroid nodules. The greatest experience has been obtained with the diagnostic use of BRAF mutation, which is strongly specific for malignancy when detected using well-validated techniques. The strongest diagnostic result can be obtained testing FNA samples for a panel of mutations that typically involves BRAF, RAS, TERT, RET/PTC, and PAX8/PPAR γ . Finding any of these mutations in a thyroid nodule provides strong indication for malignancy and helps to refine clinical management for a significant proportion of patients with indeterminate cytology.

What we know today about the diagnostic value of molecular markers it is reported in the Revised Management Guidelines for Patients with Thyroid Nodules and Differentiated Thyroid Cancer, recently published

by the ATA (7). These statements suggest to consider gene markers such as RET/PTC, BRAF, PAX8/PPAR γ , RAS and in the pre-operative setting of patient whose cytology has indeterminate result at FNAC.

The use of these and other emerging molecular markers is expected to ameliorate significantly the accuracy of cancer diagnosis in thyroid nodules. However other prospective studies are needed to identify more accurate molecular markers. Finally, the knowledge of these molecular pathways has permitted the development of new targeted therapies for aggressive TC (111,112).

The most controversial area of TC research deals with the distinction of FAs from FVPTCs that are strongly connected cancers, whose differential diagnosis is arduous. Up to now, molecular markers have been used to better characterize follicular-derived TC hoping that some of these markers will be helpful to define indeterminate FNA samples.

Some genes, earlier involved in TC pathogenesis, have been identified as able to differentiate FVPTCs from FAs. The expression of CTGF protein was significantly upregulated in TC tissues with respect to benign thyroid tumors (113) and CTGF mRNA expression was significantly different in FAs or FVPTCs, confirming that CTGF could be implicated in the malignancy of TCs. Moreover, in FVPTCs it was shown an overexpression of ITGAV and ITGB1, belonging to the integrin superfamily, as earlier reported in malignant TC (114,115). It was also shown that ECM protein FN1 mRNA expression was significantly increased in FVPTCs *vs.* FAs. In 1988 FN1 was demonstrated to be overexpressed in TCs by immunohistochemistry for the first time (116), and in 2004 galectin 3 and oncofetal fibronectin were indicated as potential markers for the preoperative diagnosis of TCs (117). Moreover, it was reported that FN1 expression was more strongly linked to malignancy with respect to adenomas and nonmalignant tissues (106).

Now a similar expression rate by semiquantitative, RT-PCR and immunohistochemistry is reported, even if the only statistically significant molecule was ITGB2. It has to be noted that recently FNA has been used to evaluate the chemosensitivity of primary TC cells in humans. This might ameliorate the response to treatment, as *in vitro* drug screening with human primary TC cells has a 60% positive predictive and a 90% negative predictive value of clinical response *in vivo* in the same patient. This could permit not to administer inactive drugs to patients (118,119).

In conclusion, BRAF mutation analysis alone does

not permit to ameliorate the diagnostic accuracy. In indeterminate TIR3 FNA patients, the combination of precise molecular marker expression analysis with molecular mutations evaluations could be assessed. For these reasons, a further expression analysis of specific molecular markers is necessary to draw decisive conclusions.

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Footnote

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