

Molecular Diagnostics of Fine Needle Aspiration for the Presurgical Screening of Thyroid Nodules

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Abstract: “The incidence of thyroid cancer, the most common endocrine malignancy, is rising. The two most common types of thyroid cancer are papillary and follicular” thyroid carcinomas. “Fine-needle aspiration (FNA) of thyroid nodules” can permit to detect many genetic mutations and other molecular alterations, including RAS and BRAF point mutations, PAX8/peroxisome proliferator-activated receptor (PPAR) γ and “RET/PTC rearrangements, occurring in thyroid papillary and follicular carcinomas” (more than 70% of cases), which can be used successfully to improve the diagnosis “and the management of patients with thyroid nodules”. The most extensive experience has been accumulated with “the diagnostic use of BRAF mutation”, which is highly specific for malignancy. “Testing FNA samples for a panel of mutations” that typically includes RAS, BRAF, PAX8/PPAR γ and RET/PTC could permit to achieve the biggest diagnostic impact. “The accuracy of cancer diagnosis in thyroid nodules could be improved significantly using these and other emerging molecular markers”.

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INTRODUCTION

“The diagnosis of thyroid cancer is the fastest growing among neoplastic diagnosis in the United States [1]”. Thyroid cancer accounts for 6% of women cancers and less than 3% of men cancers. It is estimated that 60,220 individuals (45,310 women and 14,910 men) will receive a diagnosis of thyroid cancer and 1,850 (810 men and 1,040 women) would be dead of it in 2013.

Even if most of thyroid cancers are sporadic in nature [2], the nuclear disasters and the resulting radiation exposure (for instance, Chernobyl, 1986), represent significant risk factors for thyroid cancer development [3, 4].

Papillary (PTC) and follicular (FTC) “thyroid cancers derive from follicular cells” and represent the majority of thyroid cancers (90%), while medullary thyroid cancers 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 [5].

Standard treatment for thyroid cancer usually includes primary surgery (“total or near-total thyroidectomy and lymph nodes dissection” if necessary), radioactive iodine (RAI) treatment (based on the tumor stage) and thyroid-stimulating hormone (TSH) suppressive therapy [6].

Follow-up consists of neck ultrasonography (US), basal and after TSH-stimulated thyroglobulin assay [6-9].

Though thyroid cancer has generally a good prognosis, however approximately 10-15% of patients with thyroid cancer have recurrences, and about 5% will develop metastatic disease not responsive to RAI, and eventually will die from this disease [10-13].

For these reasons an early diagnosis of thyroid cancer is needed in the patients who present thyroid nodules. US along with fine-needle aspiration (FNA) cytology (FNAC) is determinant in the discrimination of benign thyroid nodules from malignant nodules [6].

In order to improve FNAC accuracy in detecting malignancies, testing for oncogene mutations has been proposed [14, 15], suggesting that it improves the performance of FNA diagnosis when it is performed in indeterminate cytologies, where it permits to obtain the diagnosis in many cases. 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 [14].

This study reviews the usefulness of screening thyroid FNA samples for the presence of cancer-specific mutations and prognostication of thyroid cancer.

MOLECULAR PATHWAYS INVOLVED IN THYROID CANCER

BRAF

The activation of the mitogen-activated protein kinases (MAPK) is determinant in the carcinogenesis of PTC [16].

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Mutations in the BRAF gene, a member of the RAF family protein which binds RAS, lead to a constitutional phosphorylation of MEK and, in turn, of MAPK pathways. The exon 15 V600E mutation (T1799A) represents >90% of BRAF mutations and is found in about a half of PTC (45%). The BRAF V600E mutation is commonly linked to recurrent disease, the absence of tumor capsule and the loss of 131I avidity [16, 17]. “Other activating BRAF mutations have been evidenced in other positions” (for instance, 599 and 601), but their prevalence is definitely lower than in 600 [18, 19]. Recently targeted therapies against BRAF have been developed [20, 21].

RET

The RET (REarranged during Transfection) gene encodes a transmembrane receptor, located on chromosome 10q11.2, “whose intracellular domain contains two tyrosine kinase regions, docking sites for adaptor proteins, that, in turn, coordinate” cell differentiation, migration and proliferation [22, 23]. In PTC, chromosomal rearrangement between the C-terminal “kinase domain of RET and the N-terminal domain of” PTC can constitutively activate RET [13, 23]. To date, at least 13 types of RET/PTC rearrangements have been described, in particular RET/PTC1 and RET/PTC3 [23, 24]. Up to 40% of sporadic PTC brings RET/PTC rearrangements [24].

Recently new therapies targeting RET have been developed [25, 26].

RAS

RAS (“Rat sarcoma”) gene family encodes G-proteins that activate MAPK and PI3K/AKT pathways. Point mutations of N-RAS and “K-RAS at codon 12 or 13, and H-RAS at codon 61” are the most common [27]. Unlike BRAF and RET, RAS mutations are evidenced mostly in half of FTC and less frequently in follicular adenomas (20-40%), in PTC (10-15%), particularly “in the follicular variant of PTC” [27]. “RAS mutations are associated with tumor aggressiveness and” they are found effectively in half of anaplastic cancers and poorly differentiated cancers [27, 28].

PAX8/PPAR γ Rearrangements

Rearrangements involving PAX8 and PPAR γ 1 gene PAX8/PPAR γ rearrangements are almost exclusively found in follicular tumors (30-40% of FTC and 2-10% of follicular adenomas) being rare in non-classical PTC (<5%) [29, 30]. The development of FTC seems to involve independently the two pathways of PAX8/PPAR γ rearrangement and of RAS mutations, “as tumors with PAX8/PPAR γ rearrangement do not usually carry any RAS mutation” [30]. “Tumors associated with PAX8/PPAR γ usually carry a favorable prognosis” [31].

MOLECULAR DIAGNOSIS OF THYROID NODULES

Thyroid nodules are very common, since 1% of men and 5% of women have palpable nodule in iodine-sufficient countries. However, thyroid nodules are detectable by US “in 19-67% of randomly selected individuals” [6]. “Depending on sex, age, radiation exposure history and family history, and other factors, thyroid cancer” appears in 5-15% of

thyroid nodules [32]. Hence, the identification of the malignant nodules among the vast majority of benign nodules is important, as the major part “of thyroid nodules are benign and most cases of thyroid cancer are curable by surgery if detected early” [33]. “The standard preoperative diagnostic tool for thyroid cancer” is represented by the combination of FNA and cytological evaluation, but the cytological diagnosis is indeterminate for malignancy in 10-40% of cases [34]. “Since 2008, the general category of indeterminate cytology has been divided into three subcategories: “follicular lesion of undetermined significance”; “follicular or oncocyctic (Hürthle cell) neoplasm”; “suspicious for malignancy”. The three subcategories have a predicted probability for malignancy of 5-10%, 15-30%, and 50-75%, respectively” [35]. “Molecular testing of FNA biopsies (FNABs), in particular for BRAF, but also for a combination of markers (BRAF, RAS, RET/PTC and PAX8/PPAR γ) is not only possible but can significantly improve the accuracy of the preoperative FNA diagnosis from cytology” [36-46]. “The ability of genetic markers (BRAF, RAS, RET/PTC and PAX8/PPAR γ) and protein markers (galectin-3) to improve the preoperative diagnostic accuracy for patients with indeterminate thyroid nodules” have been proved by recent large prospective studies [40-42, 47-49]. Furthermore, it is now formally advised for indeterminate cytology “in the 2009 Revised American Thyroid Association (ATA) Management Guidelines for Patients with Thyroid Nodules and Differentiated Thyroid Cancer (Recommendation rating: C) to use molecular markers, as BRAF, RAS, RET/PTC, PAX8/PPAR γ or galectin-3” [6].

BRAF

A number of studies (Table 1, 40-44,48,50-56) have reported that “the accuracy of cytologic diagnosis of thyroid nodules is significantly improved by the molecular testing for BRAF V600E in thyroid FNA samples”. Recently, a meta-analysis of 18 studies about the results “of BRAF testing in 2766 thyroid FNA samples evidenced that among 581 BRAF-positive samples”, 580 were papillary carcinomas [57]. Only 1 “BRAF-positive sample, obtained as a research aspiration of the nodule in a surgically removed thyroid gland, appeared to be benign” [58]. Even if this case is considered false-negative, the rate of malignancy was 99.8% in FNA-tested BRAF-positive nodules. “Importantly, several studies have reported that 15-39% of BRAF-positive FNA samples had a cytology indeterminate or” nondiagnostic diagnosis, demonstrating that testing for the presence of BRAF mutation helps to establish a “definitive diagnosis of cancer in nodules with indeterminate cytology” [42, 44, 50, 59-61].

BRAF V600E mutation in PTC is an important “prognostic molecular marker” that improves FNAB diagnostic accuracy, but is complementary to cytology and US [45], as it was “significantly associated with malignant US features (solid composition, marked hypoechogenicity, irregular margin, taller-than-wide shape and the presence of” microcalcification) [62], and also with poor prognostic factors and increased tumor size [63].

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 [64].

Table 1. Studies that evaluated BRAF mutation in preoperative FNAC of thyroid nodules.

Author	Year	Markers Used				
Salvatore <i>et al.</i> [44]	2004	BRAF	RET			
Xing <i>et al.</i> [51]	2004	BRAF				
Domingues <i>et al.</i> [52]	2005	BRAF	RET			
Sapio <i>et al.</i> [53]	2007	BRAF	GALE-3			
Sapio <i>et al.</i> [54]	2007	BRAF	RET	TRK		
Pizzolanti <i>et al.</i> [50]	2007	BRAF	RET			
Nikiforov <i>et al.</i> [42]	2009	BRAF	RET	RAS	PAX8	
Moon <i>et al.</i> [55]	2009	BRAF				
Cantara <i>et al.</i> [48]	2010	BRAF	RET	RAS	TRK	PAX
Moses <i>et al.</i> [40]	2010	BRAF	RET	RAS		
Musholt <i>et al.</i> [41]	2010	BRAF	RET			
Ohori <i>et al.</i> [43]	2010	BRAF	RET	RAS	PAX	
Marchetti <i>et al.</i> [56]	2012	BRAF				

A perspective study [65] published in 2012 evaluated the importance “of US-guided FNAB in the diagnostic assessment of nodules with/without clinical/US features suggestive for malignancy and investigated the additional contribution of testing BRAF V600E mutation in the detection of differentiated thyroid cancer. Thyroid cytoaspirates were performed in 1856 patients (in 2421 nodules at least 4 mm in diameter) who underwent cytological evaluation and biomolecular analysis. A high positive predictive value and specificity for the diagnosis of malignant lesions was shown by cytology. The presence of the BRAF V600E mutation was found in 115 samples, 80 of which were also cytologically diagnosed as” PTC. The diagnostic value of cytology was significantly enhanced by testing the presence of BRAF mutation, “increasing FNAB diagnostic sensitivity for malignant nodules by approximately 28%” [65].

“A single-center, retrospective review of all patients who had initial thyroidectomy for histologic PTC during 2010” was published by Howell *et al.* [66]. “The correlation between the presence of central compartment lymph node metastasis (CLNM) and available preoperative clinical parameters, including tumor size, gender, age, and BRAF mutation status” was evaluated. “The BRAF V600E mutation was the only independent predictor of CLNM in PTC” among the commonly used clinical parameters available preoperatively and “could be used to guide the extent of initial surgery” [66].

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, taller-than-wide shape and absent halo [67]. 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% [67].

The conclusions of a more recent metaanalysis are that the BRAF mutation has presented extraordinary average values of specificity (97.9%) and positive predictive value (99.9%) resulting from the occurrence of only seven false positive results identified in three investigations [58, 68, 69], among the 2800 malignant and benign lesions used in the 26 investigations including BRAF. The different methods used in the detection of marker do not seem to be a disadvantage, because of the fact that they present similar results [45-50, 57-70] (Table 2, from Rodrigues *et al.* [71]).

RET/PTC

Testing for RET/PTC rearrangements can be helpful to diagnose thyroid cancer. “The preoperative diagnosis of thyroid nodules can be improved by the detection of RET/PTC in thyroid FNA samples, in particular in those samples that are indeterminate by cytology or have an insufficient amount of cells for cytologic evaluation” [38, 44, 50]. In 2 prospective studies [42, 48], it was demonstrated after surgery that all 16 thyroid nodules positive for RET/PTC were papillary carcinomas, and many of those nodules were indeterminate by cytology. In another recent study [40] 5 of 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 [72].

The conclusions of a more recent metaanalysis [71] are that the RET/PTC has presented average values of specificity (18%) and positive predictive value (87%).

RAS

The importance of RAS mutation detection lies in the fact that it is considered a marker of “the follicular variant of

Table 2. Several markers used in the preoperative evaluation of FNA from thyroid nodules. From Rodrigues *et al.* [71].

Marker	Average SN	Average SP	Average PV +	Average PV -	Average AC
<i>BRAF</i> mutations	52.35	97.92	99.85	51.62	70.54
<i>RET</i> rearrangements	18.20	88.73	87.00	59.60	55.30
<i>RAS</i> mutations	23.00	97.20	82.20	63.20	65.00
<i>PPARγ</i> rearrangements	20.00	100.00	100.00	46.00	60.00

SN, sensibility; SP, specificity; PV +, predictive positive value; PV -, predictive negative value; AC, accuracy.

papillary carcinoma”, which is most difficult to diagnose, especially by FNAC. “The second most common mutation type detected in consecutive FNA samples from thyroid nodules” is RAS mutation [73].

A retrospective study on 341 patients with thyroid cancer showed an association between malignancy and N-RAS mutation, as well as with tissue inhibitor of metalloproteinases-1 and FNAC result [74]. In several prospective studies [42, 48], “RAS mutation had a 74-88% positive predictive value for malignancy. RAS mutations were identified in those tumors difficult to diagnose by cytology alone, as follicular variant of papillary carcinoma and follicular carcinoma. The diagnosis of some RAS-positive nodules was of benign follicular adenomas”, formally contributing to a false-positive rate. However, it seems that “RAS-positive follicular adenomas are precursor lesions for RAS-positive follicular carcinomas or follicular variant of papillary carcinomas, and RAS mutation apparently predisposes the well-differentiated cancer to dedifferentiation and a more aggressive behaviour” [75-80].

In a more recent meta-analysis [71] RAS mutations has presented average values of specificity (23%) and positive predictive value (82%).

PAX8/PPAR γ

The detection of “the presence of PAX8/PPAR γ rearrangement in a follicular lesion is not completely diagnostic for malignancy by itself, but it should lead the pathologist to perform an exhaustive search for vascular or capsular invasion. The invasion is found in many PAX8/PPAR γ -positive follicular tumors after examination of the entire capsule in multiple histologic levels”, although it may not be seen at the beginning [30, 81, 82].

PAX8/PPAR γ rearrangement can be detected in samples obtained from thyroid FNA, and this is usually correlated with the presence of malignancy, although only few positive cases were evidenced so far in prospective studies [42, 43].

Recently, Ferraz *et al.* have shown also the feasibility of testing for PAX8/PPAR γ rearrangements from routine FNA [72].

In a more recent meta-analysis [71] PAX8/PPAR γ mutations have presented average values of specificity (20%) and positive predictive value (100%).

MULTIPLE TESTING

“The sensitivity of malignant diagnosis in FNA thyroid nodules increased from 44% to 80%, comparing cytology

alone to cytology combined with molecular testing for BRAF, RAS, RET/PTC and PAX8/PPAR γ [42]. The detection of any mutation was a strong predictor of malignancy, as 31/32 (97%) of mutation-positive nodules were diagnosed as malignant after surgery while only 1/32 nodule (3%) was a RAS-positive follicular adenoma” [42]. In particular, molecular testing helped to identify 15/21 (71%) malignant nodules after surgery in the indeterminate group of FNA samples, “strongly indicating that molecular testing could help to improve the diagnosis of indeterminate FNAs” [42]. “Another study [48] demonstrated that the sensitivity of malignant diagnosis in FNA increased from 60%, in the case cytology was used alone, to 90%, when cytology was combined with molecular testing for BRAF, RAS, RET, TRK, and PPAR γ mutations [48]. Similarly to the previously reported study, the presence of a mutation was a strong predictor of malignancy, as the detection of mutations was associated with cancers in 91% of the cases (61/67 mutation-positive cases) and with benign follicular adenoma in 9% of the cases (6/67 mutation-positive cases). The prospective evaluation of the clinical utility of preoperative testing for these markers is still on-going” [48].

CONCLUSION

Many genetic mutations and other molecular alterations occurring in PTCs and FTCs can be detected in FNA of thyroid nodules, and can be used successfully to “improve cancer diagnosis and the management of patients with thyroid nodules”. In particular, experience has been accumulated with the diagnostic use of BRAF mutation, strongly specific for malignancy when detected using well-validated techniques. Testing FNA samples “for a panel of mutations that typically includes BRAF, RAS, RET/PTC, and PAX8/PPAR γ ” can permit to achieve the biggest diagnostic impact. 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 “Revised Management Guidelines for Patients with Thyroid Nodules and Differentiated Thyroid Cancer, recently” published by ATA, report “the accumulation of knowledge on diagnostic use of molecular markers” [6]. The guidelines recommend “the use of molecular markers, as BRAF, RAS, RET/PTC, and PAX8/PPAR γ , for patients with indeterminate FNAC to help guide management”. “The use of these and other emerging molecular markers could improve significantly the accuracy of cancer diagnosis in thyroid nodules” [57]. However other prospective studies are needed to identify more accurate mo-

lecular markers. Finally the knowledge of these molecular pathways has permitted the development of new targeted therapies for aggressive thyroid cancer [83, 84].

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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ABBREVIATIONS

PTC	=	Papillary thyroid cancer
FTC	=	Follicular thyroid cancer
RAI	=	Radioactive iodine
TSH	=	Thyroid-stimulating hormone
US	=	Ultrasonography
FNA	=	Fine-needle aspiration
FNAC	=	FNA cytology
PPAR γ	=	Peroxisome proliferator-activated receptor
MAPK	=	Mitogen-activated protein kinase
FNAB	=	FNA biopsy
ATA	=	American Thyroid Association
CLNM	=	Central compartment lymph node metastasis

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