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Clinical Application of Molecular Testing of Fine-needle Aspiration Specimens in Thyroid Nodules

Linwah Yip, MD[a] and **Robert L. Ferris, MD, PhD**[b,*]

[a]Endocrine Surgery and Surgical Oncology, Department of Surgery, University of Pittsburgh, 200 Lothrop Street, Pittsburgh, PA 15213, USA

[b]Head and Neck Surgery, Department of Otolaryngology, University of Pittsburgh Cancer Institute, University of Pittsburgh, 200 Lothrop Street, Pittsburgh, PA 15213, USA

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INTRODUCTION

Thyroid cancer is the most common endocrine malignancy, its incidence is steadily growing, and it is currently the fifth most common cancer diagnosed in women.[1] Thyroid cancer is typically diagnosed during the evaluation of thyroid nodules, which are highly prevalent in the general population. The incidence of nodule detection has been increasing as the population ages, and as high-resolution diagnostic imaging is increasingly being used.[2] Although most thyroid nodules are benign, the challenge is to accurately and effectively identify malignant nodules. As a standard diagnostic approach, current evidence-based guidelines recommend ultrasonography evaluation with ultrasonography-guided fine-needle aspiration biopsy (FNAB) of thyroid nodules for cytologic examination.[3,4] However in approximately 25% of nodules, the cytology is indeterminate, which limits the clinical management.[5] Several molecular testing techniques have been investigated in an attempt to improve diagnostic accuracy. This article focuses on the diagnostic utility of testing for somatic mutations and rearrangements commonly found in thyroid cancer, discusses how preoperative testing can affect operative management, and examines how recently introduced technologies such as next-generation sequencing (NGS) can further expand the diagnostic capability of preoperative FNAB.

Before the routine use of thyroid nodule FNAB, malignancy was found in only 14% of resected thyroid glands.[6] In a recent meta-review, ultrasonography guidance improved diagnostic sensitivity of FNAB to 95%, but the specificity remained low at 47%.[7] When FNAB cytology has adequate cellularity, the results are classified into one of 3 categories: benign, malignant, and indeterminate. FNAB results that are benign or positive for malignancy are often accurate, with false-negative and false-positive rates of 3% to 4% and 1% to 2%, respectively.[8] However, 13% to 40% can be classified as indeterminate, which

*Corresponding author. ferrrl@upmc.edu.

represents the limitations of cytologic analysis.[3] Indeterminate FNAB results have been further subdivided by the National Cancer Institute in the widely accepted 2007 Bethesda Classification System (Table 1) into the following 3 categories: (1) suspicious for malignancy, (2) follicular neoplasm (FN), and (3) follicular lesion of undetermined significance (FLUS)/atypia of undetermined significance (AUS).[9] The suspicious category represents 3% of all FNAB results, and up to 75% prove to be malignant. Morphologic criteria for an FN result predominantly include a hypercellular aspirate of follicular cells indicating follicular proliferation with or without microfollicles, and the differential diagnosis encompasses follicular adenoma (FA), follicular thyroid carcinoma (FTC), or follicular variant of papillary thyroid carcinoma (fvPTC).[9] The malignancy rate is lower (up to 26%), but cytology results indicating FN currently require the patient to undergo at least thyroid lobectomy for definitive diagnosis.[3] The FLUS/AUS category is the most heterogeneous and represents those cytology specimens that are neither benign nor malignant, but may have a degree of cellular and/or architectural atypia that does not meet morphologic criteria for being FN or suspicious.[9] A repeat FNAB may lead to a benign cytology result in up to 40% of nodules[10,11]; however, the malignancy risk is as high as 16% and diagnostic surgery may still be necessary.[8] Thus, many patients undergo surgical resection of benign disease resulting in potentially avoidable morbidity as well as suboptimal use of limited health care resources. Improved diagnostic accuracy of FNAB cytology evaluation ideally obviates diagnostic surgery and guides the appropriate extent of initial surgery.

MOLECULAR CAUSES OF THYROID MALIGNANCIES

The identification of molecular pathways known to be implicated in thyroid carcinogenesis has significantly expanded options for developing diagnostic adjuncts. The identification of these somatic mutations and rearrangements has helped to further elucidate the specific genetic alterations that occur during the progression from follicular hyperplasia to well-differentiated carcinoma, and much attention has been directed toward elucidating the molecular signature of the 3 most commonly encountered histologic subtypes, which are difficult to differentiate based on FNAB alone: fvPTC, FTC, and FA.[12] Although the diagnosis of histologic variants such as poorly differentiated and anaplastic thyroid cancers is typically straightforward, identification of markers that may herald development of aggressive biological behavior also contributes to improved preoperative risk stratification.

Papillary Thyroid Carcinoma

The mitogen-activated protein kinase (MAPK) pathway alters signaling pathways, induces cell cycle progression, and is typically activated by receptor tyrosine kinases. However, MAPK-mediated tumorigenesis can also be modulated by alternate oncogenic mechanisms such as methylation, chemokine activation, and alterations in components of the tumor microenvironment.[13] Several mutations comprising components of the MAPK pathway have been documented in the development of papillary thyroid carcinoma (PTC) including *RAS* and *BRAF* mutations, and *RET/PTC* rearrangements.[14] Alterations in this oncogenic pathway have been shown in many different malignancies and targeted therapies inhibiting this pathway have been used effectively for a variety of tumor types (eg, Hodgkin

lymphoma, glioblastoma multiforme, squamous cell carcinoma, non–small cell lung cancer, and melanoma).

The most common genetic mutation for PTC is an activating point mutation in the *BRAF* gene, occurring in approximately 45% of all PTC.[14] The mutation that leads to substitution of valine for glutamate at residue 600 (*BRAF* V600E) has been implicated in other tumors including melanoma and, less frequently, colorectal adenocarcinoma. Transgenic mice with thyroid-specific *BRAF* V600E expression develop aggressive and radioiodine-resistant PTC.[15,16] The mutation is more commonly seen in the classic version of PTC and the tall-cell variant, but can also be identified in anaplastic carcinoma, poorly differentiated thyroid cancer, and primary thyroid lymphomas. More importantly, FTC and benign lesions do not carry this mutation, thereby making it a specific diagnostic marker for PTC. In a study of more than 4500 cytology samples, all *BRAF* V600E–positive FNAB specimens were histologic malignancies.[17] *BRAF* K601E is the second most common BRAF mutation identified in thyroid cancer and is more likely to be associated with fvPTC.[18]

RAS mutations are found in approximately 10% to 20% of PTC tumors, and are more commonly encountered in fvPTC and FTC.[19] However, *RAS* mutations can be found in the full spectrum of thyroid neoplasms ranging from benign follicular hyperplasia and FA to anaplastic carcinomas.[14] The *RAS* gene encodes for G proteins that are bound to cell membrane receptors and, when stimulated by extracellular signals, result in cellular dysregulation. Activated *RAS* is bound to guanosine triphosphate (GTP), which is tightly mediated by intrinsic *RAS*-regulated GTPase, leading to the inactive GDP-bound *RAS*. Mutations within *RAS* cause constitutive activation either by inactivating the autocatalytic GTPase function (codon 12 or 13) or increasing the binding affinity for GTP (codon 61). The 3 *RAS* gene isoforms (*NRAS*, *KRAS*, and *HRAS*) can activate both the MAPK and phosphatidylinositol 3 kinase (PI3K) pathways.[13] Mutations resulting in *RAS* activation have been shown in 20% to 25% of all human tumors.[13]

RET proto-oncogene point mutations are classically identified in medullary thyroid carcinoma (MTC) and, when genomic, are associated with MEN2 and familial MTC. However, the role of *RET* rearrangements in PTC has been well documented with more than 10 different types of translocations described that are identified in 10% to 20% of PTC.[20,21] The 3′ tyrosine kinase portion of the *RET* gene fuses with the 5′ of a different gene resulting in ligand-independent dimerization and constitutive activation of effector genes in the MAPK and PI3-AKT pathways. The 2 most common fusion proteins are *RET/PTC1* and *RET/PTC3*, which are paracentric versions with the 5′ domain of 2 genes on chromosome 10: *CCDC6* and *NCOA4*, respectively.[20] A higher incidence of *RET/PTC* rearrangements are seen in patients with PTC with a previous history of radiation exposure (50%–80%) or in young patients (40%–70%).[22–24] *RET/PTC1*-positive tumors show either classic papillary architecture or diffuse sclerosing features, whereas *RET/PTC3* is associated with the solid variant. All of the *RET/PTC* tumor subtypes possess a high rate of lymph node metastases.[25] Using highly sensitive detection methods, nonclonal *RET/PTC* rearrangements have also been identified in up to 45% of benign nodules.[26]

TRK rearrangements are gene fusions of the *NTRK1* receptor tyrosine kinase gene on chromosome 1q22, which is one of at least 3 different genes described to date. These rearrangements can also be found in less than 5% of PTC.[12]

FTC and FA

Genetic alterations in the PI3K-AKT pathway are common in FTC and FA.[13,27] Germ-line mutations in the *PTEN* gene are responsible for Cowden syndrome, which is characterized by benign and malignant follicular thyroid tumors. Increased expression and activation of AKT was reported in FTC and poorly differentiated thyroid cancer, but has also been observed in all subtypes.[28] Somatic point mutations in *PTEN* are identified in ~10% of FTC, but gene methylation resulting in reduced expression levels may be a more frequent mechanism of *PTEN* loss in follicular thyroid lesions.[13] As previously discussed, *RAS* mutations can also activate the PI3K-AKT pathway, and are found in 40% to 50% of FTC and 20% to 40% of FA.[19] Although benign follicular lesions carrying the *RAS* mutations may be precancerous lesions, the identification of *RAS* is nonspecific for histologic malignancy.

A gene rearrangement leading to the fusion of the thyroid-specific paired domain transcription factor *PAX8* and the peroxisome proliferator-activated receptor gene *PPARγ*, which plays an important role in lipid metabolism, was discovered in FTC in 2000.[29] The *PAX8/PPARγ* rearrangement results in overexpression of the fusion protein, but the carcinogenic mechanism of action is unclear. *PAX8* plays an essential role in thyrocyte development, as well as in the gene expression of the sodium-iodide symporter, thyroglobulin, and the thyroid-stimulating hormone receptor.[30] The fusion protein antagonizes the action of *PPARγ* via a dominant-negative inhibition that has been shown to be a causative agent in FTC in vitro and in vivo tumorigenesis.[31] However, the exact carcinogenic consequence of the *PAX8/PPARγ* fusion protein remains theoretic. *PAX8/PPARγ* translocation is found in 30% to 40% of classic FTC, 2% to 10% of FA, and rarely in fvPTC.[32] *PAX8/PPARγ*-positive FA tends to have positive immunohistochemical staining for markers more consistent with FTC, but does not meet histologic criteria for malignancy. Much like *RAS*-positive FAs, *PAX8/PPARγ*-positive FA may represent carcinomas in situ. *PAX8/PPARγ*-positive FTCs tend to occur in young patients with tumor characteristics that have solid patterns and vascular invasion.[32]

Poorly Differentiated Thyroid Carcinomas

The progression from differentiated thyroid cancer to poorly differentiated or anaplastic thyroid cancer is less well explicated, and could theoretically be caused by either accumulation of multiple genetic alterations resulting in oncogenic amplification, or coexistent MAPK and PI3K-AKT pathway dysregulation that accelerates the genetic alterations that promote tumor growth. Poorly differentiated thyroid carcinomas (PDTC), although uncommon, display more aggressive behavior than well-differentiated thyroid carcinomas, but are less aggressive than the undifferentiated or anaplastic forms. Histology, immunohistochemistry, and molecular genetic tests reinforce the diagnosis of PDTC. Given the aggressiveness of PDTC and the poor survival rates in patients who undergo surgery alone, a multimodality treatment approach is required.

Although rearrangements are rarely identified in PDTC, point mutations found in differentiated thyroid cancer are well-represented. *BRAF* V600E is present in ~15% to 25% of patients with PDTCs but is rarely found in PDTCs arising from FTC. *RAS* gene alterations (commonly point mutations at *HRAS* codon 12 or 61 and *NRAS* at codon 13) are present in 25% to 35% of PDTCs.[12] *RAS*-induced chromosomal instability may predispose to tumor dedifferentiation, perhaps explaining the increased prevalence of mutant *RAS* in anaplastic thyroid carcinomas. However, mutant *RAS* is unlikely to be solely capable of driving tumor dedifferentiation, given its high prevalence (45%) in patients with differentiated thyroid cancer and in those with benign thyroid adenomas.[12] In contrast, Garcia-Rostan and colleagues[33] stated that histologic dedifferentiation is not necessarily driven by *BRAF* or *RAS* mutations individually, but rather represents the cooperation of multiple genetic alterations that likely stimulate dedifferentiation.

Inactivating point mutations of *TP53* are rarely associated with differentiated thyroid cancers; however, they are highly prevalent (25%–30%) in patients with PDTC and ATC.[12,13] Because *p53* is known as the guardian of the genome, unlike *RAS* and *BRAF* gene alterations, which regulate proliferative signals, *p53* mutations possess an important function in triggering tumor dedifferentiation and evolution to PDTC and ATC. Other gene mutations that are associated with tumor dedifferentiation include point mutations in *PIK3CA* (10%–20%), *CTNNB1* encoding beta-catenin (10%–20%), and *AKT1* (5%–10%).[12]

BIOMARKERS USED FOR TUMOR DETECTION AND PROGNOSTICATION

Several prospective and retrospective studies have shown that the diagnostic accuracy of FNAB can be significantly improved using modern molecular detection techniques to identify genetic alterations. In addition, although most patients with differentiated thyroid cancer fare well, biomarkers have also been shown to provide prognostic information that can be used to guide further management.

Molecular Testing of FNAB Specimens

BRAF—Most studies of biomarker detection in FNAB specimens have focused on the *BRAF* V600E mutation. In a 2009 review, Nikiforova and Nikiforov[14] reported on testing for *BRAF* in 2766 samples from 9 prospective fine-needle aspiration (FNA) studies, 7 retrospective FNAB studies, and 2 studies of research FNAB performed on postoperative thyroid specimens (Table 2). In this meta-analysis, all 580 *BRAF*-positive clinical FNAB samples studied prospectively and retrospectively were positive for papillary carcinoma, and there was only 1 reported false-positive sample, obtained by research aspiration of the nodule in a surgically removed thyroid gland, resulting in a false-positive rate of 0.2%.[14] *BRAF* V600E mutation can occasionally be detected in false-negative benign FNAB results from histologic malignancies; however, *BRAF* V600E is less common overall than other gene alterations in cytologically indeterminate FNAB specimens.[18,34] Regardless of cytology category, when preoperative *BRAF* V600E is detected in FNAB testing, a diagnosis of thyroid cancer should be suspected. *BRAF* K601E is less frequently detected, but is associated with indeterminate cytology and indolent histology. In a series of 29

indeterminate FNAB specimens with positive *BRAF* testing, 8 (28%) were *BRAF* K601E with histologic malignancies confirmed in all (100%). Only 1 of the 8 malignancies was a solid variant PTC, whereas the remaining 7 were histologic fvPTC.[18]

RAS—*RAS* mutations are found in both benign and malignant follicular thyroid neoplasms and are the most frequent mutations detected in cytologically indeterminate FNAB results because of the association with FA, FTC, and fvPTC histologies. In a series of 68 *RAS*-positive FNAB specimens, 93% were cytologically indeterminate. The rate of histologic malignancy was 83% and included 46 fvPTC, 4 FTC, 1 MTC, and 1 anaplastic cancer. Most fvPTC was encapsulated, and lymph node metastasis was uncommon.[35] In another study including 97 indeterminate FNAB specimens, *NRAS*-positive results on multivariate analysis also added diagnostic accuracy to preoperative cytology.[36] Although *RAS* positivity is not 100% predictive of malignancy, detection increases the risk to 80% to 85%, which is often high enough to alter initial surgical management to total thyroidectomy for patients who may otherwise require 2-stage thyroidectomy based on indeterminate cytology.[34] Even if histology is benign, there is the potential for malignant transformation associated with a *RAS*-positive FA and surgical resection may be a reasonable treatment option.

RET/PTC—Among differentiated thyroid cancers, the *RET/PTC* rearrangement is typically associated with PTC. In a retrospective analysis comparing patient-matched FNA and post-thyroidectomy specimens, a *RET/PTC* fusion transcript was present in 50% of the FNA samples, all of which were histologically proven PTC in the surgically removed thyroids.[37] No false-positive results were reported in this study. The results confirmed that *RET/PTC* is a highly specific biomarker for the diagnosis of PTC. In addition, the data suggested that molecular investigation was most informative for aspirates that would otherwise have been nondiagnostic. In 2 of the 6 histologically proven PTCs that had insufficient aspirate for diagnosis by cytologic examination, the correct diagnosis was made by screening for *RET/PTC* on the FNA samples. Of the 15 indeterminate FNAB that were eventually diagnosed as PTC after surgery, 9 were positive for *RET/PTC*. However, if considering *RET/PTC* alone as the diagnostic biomarker, only 50% of the PTC were identified based on the use of molecular diagnostics with *RET/PTC* as the biomarker. In addition, in this series, using both cytologic analysis and *RET/PTC* detection allowed an increased diagnostic yield, from 12 cases definitively diagnosed by cytologic examination alone to 23 cases diagnosed by cytology and molecular marker amplification.[37]

Panel testing—Individual marker testing lacks sufficient specificity and the optimal diagnostic accuracy with DNA-based testing can be achieved with a panel of markers. Testing FNAB specimens for a panel of mutations including *BRAF*, *RAS*, *RET/PTC*, and *PAX8/PPARγ* was first evaluated in 2 independent single-institution series and was shown to be highly associated with thyroid cancer in 146 cytologically indeterminate FNAB specimens with histologic correlation.[38,39] In these studies, 49% of nodules with an identified mutation had one of the non-*BRAF* mutations, and 97% were diagnosed with histologic thyroid cancer. The largest, prospective study done thus far with cytologic, molecular, and histologic correlation included 513 consecutive cytologically indeterminate FNAB specimens.[34] The malignancy rate was 24%. When *BRAF*, *RET/PTC1*, *PTC3*, or

PAX8/PPARγ were detected, the risk of malignancy was nearly 100% regardless of cytology category. *RAS* mutations were detected in 73% of the molecular-positive FNAB specimens, and histologic thyroid cancer was diagnosed in 85%. For indeterminate FNAB, the mutation panel had a sensitivity 61%, negative predictive value 89%, specificity of 98%, and positive predictive value (PPV) 89%.[34]

The primary benefit of the panel was to improve the PPV of preoperative testing (Table 3). For patients with molecular-positive FNAB results, the overall risk of malignancy was 89% and total thyroidectomy should be considered as the initial surgical procedure. In a series of 471 patients with FNAB results classified as AUS/FLUS or FN, prospective molecular testing using the mutation panel was associated with a 2.5-fold reduction in 2-stage thyroidectomy for histologic clinically significant thyroid cancer.[40] Furthermore, in hypothetical decision-tree modeling, the significant reduction in unnecessary surgeries offset the added costs of molecular testing and resulted in distributed cost savings.[41] Negative mutation panel results may also reduce malignancy risk to allow modifications to current management algorithms for selected patients. For example, mutation-negative AUS/FLUS nodules have a 5.9% risk of malignancy with no malignancies diagnosed in nodules smaller than 1.8 cm[42] and sonographic surveillance may be an option for patients with this subset of small nodules.

Prognostication Using Biomarkers

Although most well-differentiated thyroid carcinomas have indolent behavior and are easily cured by surgical removal, a minority of tumors can be highly aggressive and difficult to treat. The current classifications rely on clinical characteristics (ie, age, extrathyroidal extension, size, and tumor grade) but do not predict the behavior of a neoplasm with any great accuracy. Mutation testing has also shown some promise in this regard, which may also help guide the initial extent of surgical management.

BRAF V600E mutation has been well studied as a prognostic biomarker for PTC. In a review by Xing,[43] *BRAF* mutations correlated with aggressive tumor characteristics such as extrathyroidal extension, advanced tumor stage at presentation, and lymph node or distant metastases. The mutation was further shown to be a poor prognostic indicator when positive in FNAB samples. *BRAF* V600E has also been shown to be an independent predictor of tumor recurrence, even in patients with early stage disease. Elisei and colleagues[44] examined 102 patients with PTC over a median of 15 years, and the *BRAF* V600E mutation was an independent risk factor for tumor-related death. More recently, Xing and colleagues[45] examined a multi-institutional and retrospective series of 1840 patients with PTC with median follow-up of 33 months, and observed an association between *BRAF* V600E and increased disease-specific mortality (hazard ratio, 2.66; 95% confidence interval, 1.3–5.4; $P<.001$). However, in multivariate analysis, the effects of *BRAF* did not seem to be independent of extent of disease at presentation including presence of lymph node metastasis, extrathyroidal extension, and distant metastasis. Therefore the utility of *BRAF* V600E testing may be greatest during preoperative planning and in determining the extent of initial surgery. Given the association with lymph node metastasis, lymph node mapping by high-resolution ultrasonography should be performed before initial surgery.

In the absence of clinical or sonographic lymph node involvement, controversy exists as to whether or not a prophylactic central compartment lymph node dissection (CCND) should be performed.[46] In single-institution small studies, prophylactic CCND reduced postoperative thyroglobulin levels and may decrease locoregional recurrence rates, although no study has yet shown a reduction in disease-specific mortality.[47–50] In addition, a higher rate of postoperative morbidity, including temporary hypocalcemia, has been observed even when the procedure is performed by high-volume surgeons.[49] *BRAF* V600E–positive PTC has a higher risk of central compartment lymph node metastasis and, in multivariate analysis, *BRAF* remains a potent preoperative predictor of nodal disease.[51,52] Thus, *BRAF* V600E may be one way to identify patients who would best benefit from prophylactic CCND.

Several studies have also reported that *BRAF* V600E in papillary thyroid microcarcinomas (PTMC) correlates with higher rates of both extrathyroidal tumor extension and cervical lymph node metastasis.[53,54] Most PTMC are indolent tumors that are incidentally discovered during the removal of presumed large, benign neoplasms, and are almost universally cured by surgical resection. However, a subset of PTMC can behave aggressively, leading to recurrence and mortality. In a molecular-pathologic score derived from a cohort of PTMC and then validated in an independent cohort, *BRAF* V600E along with histopathologic features including fibrosis, superficial location, and intraglandular tumor spread/multifocality could predict PTMC at higher risk for aggressive behavior.[55]

The role of *RAS* mutation as a prognostic biomarker has not been as clearly delineated. As previously mentioned, this tumor is found in both benign and malignant follicular processes, making it difficult to use it for prognosis. However, there is evidence to suggest that an FA that is *RAS* positive is more likely to be a carcinoma precursor or carcinoma in situ. Some studies have suggested a significant correlation between the *RAS* mutation and metastatic potential of FTC that may be caused by the role of *RAS* mutation in tumor dedifferentiation and progression to anaplastic carcinoma.[12] In a series of 91 tumors followed for a median of 14 years, *RAS* mutation correlated with distant metastasis and a significantly higher mortality.[56] Overall, because of its presence in benign and largely indolent tumors, the *RAS* mutation has not been a consistently reliable, prognostic biomarker.

Unlike PTCs with *BRAF* and *RAS* mutations, those with *RET/PTC* genetic rearrangement have shown a favorable prognosis.[57] They have been found to have a lower probability of tumor dedifferentiation and metastasis, but may be associated with a higher risk of lymph node involvement.

Genetic Analysis of Multifocal PTC

Multifocal PTC can represent either intraglandular spread from a single clonal cancer or multiple synchronous primary cancers. Using a series of 60 multifocal PTCs with 2 to 4 discrete tumor foci, genetic mutations, and histopathologic characteristics, we tested for *BRAF*, *NRAS*, *HRAS*, and *KRAS* point mutations and *RET/PTC1* and *RET/PTC3* rearrangements.[58] As expected, *BRAF* mutations were found in 43% of tumors, *RAS* in 27%, and *RET/PTC* in 2%. We identified 4 subgroups of these PTCs: (1) 2 foci containing different mutations (30%); (2) 1 tumor with a mutation and another without mutations

(32%); (3) all cancers containing the same mutation (25%); and (4) absence of detectable mutations in all cancers (13%). We concluded that up to 60% of multifocal PTC were likely synchronous primary tumors because 30% of cases had 2 different mutations likely representing separate clonal cancers and, in an additional 30% of cases, one cancer had an identified mutation whereas the other did not. On histopathology, these multifocal cancers were also located in different lobes, showed distinct growth patterns, and showed no evidence of peritumoral dissemination.[58]

TECHNIQUES FOR BIOMARKER DETECTION

Testing for the various biomarkers is based on the type of mutation. For point mutations, *BRAF* and *RAS*, many different techniques have been shown to be effective: polymerase chain reaction (PCR) and Sanger sequencing, pyrosequencing, real-time PCR amplification with post-PCR melting curve analysis, and allele-specific PCR.[59]

In the Sanger method, the DNA strand to be analyzed is used as a template and a DNA polymerase is used to generate complimentary strands using primers. Four different PCR reaction mixtures are prepared, each containing a certain percentage of dideoxynucleoside triphosphate (ddNTP) analogues to one of the 4 nucleotides, which, when encountered during synthesis, terminates the reaction. Each of the 4 PCR reactions then has a mixture of different lengths of DNA strands, all ending with the nucleotide that was dideoxy labeled for that reaction. Gel electrophoresis is then used to separate the strands of the 4 reactions, in 4 separate lanes, and to determine the sequence of the original template based on what lengths of strands end with what nucleotide. The 4 reactions are then combined and applied to a single lane of a gel. Laser-generated chromatograms are then generated from which the template DNA sequence can be determined.

The pyrosequencing method allows sequencing of a single DNA by synthesizing the complementary strand 1 base pair at a time and detecting which base was added at each step. Solutions containing A, C, G, and T nucleotides are added sequentially and removed from the reaction. Light is produced only when the nucleotide solution complements the first unpaired base of the template, which results in chemiluminescent signals and elucidates the template sequence.

Real-time PCR quantifies the amount of a particular gene present by detecting fluorescence once double-stranded DNA is amplified. As the reaction proceeds, the point at which a certain threshold of fluorescence is reached indicates how much of the gene of interest is present in the sample.

In allele-specific PCR, a primer is chosen that contains the mutation in the gene of interest, which therefore only initiates the PCR reaction if the altered gene is present.

For the chromosomal rearrangements, *RET/PTC* and *PAX8/PPARγ*, the testing can only be reliably performed on fresh or immediately frozen FNAB samples.[34] Reverse transcriptase PCR is used in either real-time mode or in a conventional fashion to detect the mutated RNA present. The sensitivity should not be too high, because the tissues acquire more chromosomal rearrangements as they age and become damaged. If testing samples fixed in

formalin or paraffin, fluorescence in situ hybridization is the assay of choice because PCR is so sensitive. However, this makes testing on fixed tissue less practical in the clinical setting.

NGS

NGS offers simultaneous massively parallel sequencing of millions of DNA sequences and may be cost-effective for detecting multiple genetic alterations. An advantage compared with conventional Sanger sequencing is that it allows simultaneous analysis of large regions of the genome and offers high sensitivity of mutation detection and quantitative assessment of mutant alleles. NGS comprises whole-genome sequencing, whole-exome sequencing, and whole-transcriptome sequencing as well as targeted sequencing of multiple specific genomic regions. Targeted NGS panels may improve routine molecular diagnostics of cancer. In thyroid nodules, such an approach may be helpful to expand the currently available diagnostic panels of several genes to enable simultaneous testing for multiple mutations. Our group recently evaluated targeted next-generation sequencing as a new approach for testing a broader spectrum of point mutations that occur in thyroid cancer.[60] Among FTCs, somatic *TSHR* mutations were identified in 22% of cases. Of 7 *TSHR* mutations identified, 5 were in malignant and 2 in benign nodules, suggesting that the location of this mutation in a thyroid nodule may have some association with thyroid cancer, particularly with follicular carcinoma. Another significant observation was the detection of *TP53* mutations in oncocytic follicular carcinomas. *TP53* mutations are known to occur with increasing frequency in dedifferentiating thyroid tumors but not in well-differentiated cancer. The high frequency of *TP53* mutations in these tumors was not caused by a sensitive detection using the NGS approach because the mutation was found at high allelic frequency. However, *TP53* mutation occurred in approximately 20% of oncocytic follicular cancers, suggesting that this tumor subset may be prone to dedifferentiation, providing a potential diagnostic and prognostic marker in this cancer type.

The high frequency of mutation detection in thyroid cancers by ThyroSeq is expected to further improve the sensitivity of cancer detection in thyroid nodules with indeterminate FNA cytology that are currently tested using more limited mutational panels. Because of the inclusion of additional gene hotspots such as within *TP53* and *TSHR*, as many as 68% of all tumor types were identified by the ThyroSeq panel to carry at least one point mutation. If combined with the detection of chromosomal rearrangements, potentially more than 80% of all thyroid cancer would be expected to have at least one detectable mutational event. Extended mutational profiling using the ThyroSeq panel revealed that 9 of 99 mutation-positive cancers (9%) contained more than one mutation. These tumors included either dedifferentiated tumors (6 anaplastic and 1 PDTC) or PTCs (n = 2), both with unfavorable prognostic features such as distant metastasis or local tumor recurrence. Most of these tumors had a combination of either *BRAF* or *NRAS* mutation, known to be an early driver event in thyroid cancer, and *TP53* and/or *PIK3CA* mutation, which are thought to be late events in tumor clone progression. The occurrence of multiple mutations has been reported before in advanced thyroid cancer and was observed in this study, suggesting that, in addition to its diagnostic utility, this comprehensive mutational panel may further contribute to preoperative thyroid cancer risk stratification by mutation testing of FNAB samples.

SUMMARY

Molecular techniques to detect mutations and rearrangements in thyroid FNAB specimens has already shown significant clinical utility in improving the preoperative diagnosis of well-differentiated thyroid carcinomas, and has been particularly useful for risk stratification of cytologically indeterminate FNAB results. With emerging new technologies that continue to broaden the spectrum of evaluable genetic alterations, the accessibility and applicability of molecular testing is likely to continue to expand. The added preoperative prognostic information can also be used to help guide extent of initial surgery, although future study is still needed to determine whether tailored operative management will improve disease outcomes.

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KEY POINTS

- Distinct genetic alterations involving the mitogen-activated protein kinase and phosphatidylinositol 3 kinase pathways characterize thyroid cancer subtypes, and can be detected in preoperative fine-needle aspiration biopsy specimens.
- Identification of these gene mutations and rearrangements can improve the diagnostic utility of preoperative testing, particularly for nodules that are cytologically indeterminate.
- Mutation testing also adds preoperative prognostic information including the identification of thyroid cancers that have a high risk of aggressive histopathologic features, which may further guide the extent of initial thyroidectomy and lymphadenectomy.

Table 1

The National Cancer Institute's suggested fine-needle aspiration (FNA) terminology and the risk of malignancy based on the cytopathologic result

FNA Result	Alternate Accepted Nomenclature	Risk of Malignancy (%)
Benign	—	<1
Atypia of undetermined significance	Atypical lesion of undetermined significance, follicular lesion of undetermined significance, indeterminate follicular lesion, atypical follicular lesion	5–15
Neoplasm	Suspicious for neoplasm, follicular neoplasm	20–30
Suspicious for malignancy	—	50–75
Malignant	—	100
Nondiagnostic	Unsatisfactory	—

Table 2

A review of all thyroid FNA studies using the BRAF mutation before 2009. The results of prospective, retrospective, and FNA studies on surgically removed thyroid specimens are shown. BRAF positivity shows an almost universal correlation with a final pathologic result of PTC

Thyroid FNA Studies	Number of Samples	BRAF Positive	Final Diagnosis in BRAF-positive Samples
Prospective studies	1814	159	PTC, 159 (100%)
Retrospective studies	685	291	PTC, 291 (100%)
FNA on thyroid specimens	267	131	PTC, 130 (99.2%) Hyperplasia, 1 (0.8%)
Total	2766	581	PTC, 580 (99.8%)

Table 3

Correlation between the results of mutational analysis in FNA samples and outcome in specific groups of indeterminate cytology

AUS/FLUS (n = 247)[a]			
	Histology Malignant (n = 35)	Histology Benign (n = 212)	Sensitivity, 63%
			Specificity, 99%
Mutation Positive (n = 25)	16 RAS (16 fvPTC)	3 RAS (3 FA)	PPV, 88%
	5 BRAF (4 fvPTC)		NPV, 94%
	1 PAX8/PPARg (1 fvPTC)		Accuracy, 94%
Mutation Negative (n = 222)	13 (11 fvPTC, 2 PTC)	209 (166 NH, 42 FA)	
FN or Hürthle Cell Neoplasm/Suspicious for FN (n = 214)[b]			
	Histology Malignant (n = 58)	Histology Benign (n = 156)	Sensitivity, 57%
			Specificity, 97%
Mutation Positive (n = 38)	2 BRAF (1 PTC, 1 fvPTC)	5 RAS (5 FA)	PPV, 87%
	29 RAS (21 fvPTC, 5 PTC, 3 FTC)		NPV, 86%
			Accuracy, 86%
	2 PAX8/PPARg (2 fvPTC)		
Mutation Negative (n = 176)	25 (16 fvPTC, 3 PTC, 6 FTC)	151 (95 HN, 56 FA)	
Suspicious for Malignant Cells (n = 25)[c]			
	Histology Malignant (n = 28)	Histology Benign (n = 24)	Sensitivity, 68%
			Specificity, 96%
Mutation Positive (n = 20)	10 BRAF (10 PTC)	1 RAS (1 FA)	PPV, 95%
	7 RAS (6fvPTC, 1 FTC)		NPV, 72%
	1 PAX8/PPARg (1 FTC)		Accuracy, 81%
	1 RET/PTC (1 PTC)		
Mutation Negative (n = 32)	9 (7 PTC, 2 fvPTC)	23 (17 HN, 6 FA)	

Abbreviation: HN, hyperplastic nodule.

[a] Molecular testing reduced observed malignant frequency from 16% to 6%.

[b] Molecular testing reduced observed malignant frequency from 27% to 14%.

[c] Molecular testing reduced observed malignant frequency from 54% to 28%.